Characterizing cellular mechanisms of cocaine-evoked synaptic plasticity in the nucleus accumbens

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Dedication

This dissertation is dedicated to my wonderful family and friends who have provided tireless support and encouragement to me throughout graduate school.

Abstract

Repeated exposure to drugs of abuse alters the structure and function of neural circuits mediating reward, generating maladaptive plasticity in circuits critical for motivated behavior. Within mesocorticolimbic dopamine circuitry, repeated exposure to cocaine induces progressive alterations in AMPAR-mediated glutamatergic synaptic transmission. During abstinence from cocaine treatment, AMPAR signaling is potentiated at synapses on nucleus accumbens (NAc) medium spiny neurons (MSNs), promoting a state of heightened synaptic excitability. Re-exposure to cocaine during abstinence, however, reverses and depotentiates enhanced AMPAR signaling, demonstrating that cocaine bidirectionally alters excitatory synaptic transmission in the NAc. Understanding the neurobiological mechanisms underlying drug-induced synaptic adaptations in the NAc could provide targets for developing strategies to reverse or offset maladaptive processes driving long-lasting vulnerability to relapse. However, the detailed cellular signaling mechanisms mediating cocaine-evoked plasticity have not been well-characterized. Using pharmacological approaches in combination with patch-clamp recordings in the NAc, we investigated the role of candidate signaling factors that mediate adaptive synaptic plasticity in the striatum. Among these, activation of group I metabotropic receptors (mGluR1/5) play a prominent role in synaptic depression at excitatory synapses, and furthermore are implicated in models of relapse to drug-seeking. Consistent with this, we found that activation of mGluR5 is necessary for cocaine-induced depotentiation of AMPAR signaling in the NAc. Downstream of mGluR1/5 receptors, mobilization of endogenous cannabinoids (eCBs) is an important factor

modulating excitatory synaptic strength. Dopamine receptors in the striatum also broadly modulate synaptic transmission at glutamatergic terminals on MSNs, and are critically engaged by drugs of abuse. Both dopamine and eCB signaling were necessary factors in the induction of cocaine-induced synaptic plasticity in the NAc, suggesting that these neuromodulators may modify the responsiveness of MSNs to alterations in glutamatergic input induced by cocaine. Finally, we examined plasticity at synapses on specific MSN cell subpopulations, demonstrating that specific dopamine receptors on distinct cell types promote specific modifications in AMPAR synaptic function following cocaine experience. These neuromodulatory signaling mechanisms may serve to gate the induction of plasticity at glutamatergic afferents on NAc MSNs by converging on common factors that control the sensitivity of MSNs to excitatory input, ultimately driving addiction-related behavior.

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Introduction and background

Drug addiction is a major public health problem in the US, affecting an estimated 8-10% of adult Americans (SAMHSA, 2015). Up to 4% of the US adult population has abused illicit psychostimulant drugs including cocaine and methamphetamine in 2015, with 900,000 of these individuals being diagnosed with a substance use disorder (SAMHSA, 2015). While the current opioid abuse epidemic might suggest that abuse of other substances including psychostimulants is a less urgent issue, national data on overdose deaths during 2010-2014 indicates that the age-adjusted rate of drug overdoses involving psychostimulants has more than doubled during the last five years (Warner et al., 2016). Each year, cocaine consistently ranked second or third among drugs involved in overdose deaths. Furthermore, cocaine use was a common factor in overdoses involving multiple drugs, which described nearly half (48%) of all overdose deaths during 2010-2014. These recent findings provide evidence that psychostimulant addiction remains a major issue in the US. Effective therapies for addiction assist individuals in achieving enduring relief from relapse. However, while several pharmacological therapies have been FDA-approved for treating opioid abuse, none currently exist for psychostimulant addiction. Developing effective treatments for addiction requires understanding the neurobiological mechanisms underpinning drug seeking behavior and the complex physiological, cognitive, and motivational aspects of addiction.

Synaptic plasticity and addiction

Over the past several decades, an extensive body of research has demonstrated that alterations in neural circuitry critical for reward-related learning, motivation, and cognition underlie key features of drug addiction (Hyman et al., 2006; Thomas et al., 2008; Volkow and Morales, 2015). These circuits naturally permit organisms to learn about their environments and adapt to important stimuli, including whether to approach sources of food or sex, or to avoid harmful situations (Everitt and Robbins, 2005). In drug addiction, neural circuitry important for reward and reinforcement undergo neuroplasticity that develops as a consequence of repeated drug experience. Over time, modifications in cells, circuits, and systems impair the selection of adaptive behavioral stimuli in favor of progressively-increasing drug-seeking and drug-taking strategies (Kalivas and Volkow, 2005). These strategies manifest clinically as persistent and recurrent drug-seeking behavior at the expense of pursuit of natural rewards (Kalivas and O'Brien, 2007). The chronic, relapsing nature of addiction is today generally accepted by the medical and scientific communities to reflect aberrant resculpting of motivational circuitry that drives drug-seeking, preventing lasting abstinence. However, few effective treatment options exist. Successful therapeutic strategies for treating drug addiction must assist individuals in maintaining abstinence, providing relief from the cycle of intoxication, abstinence, and craving that leads to relapse (Koob and Volkow, 2009). To do so will require understanding the drug-induced neurobiological alterations in brain function that undergird long-lasting vulnerability to relapse.

The nucleus accumbens: structure, function, and role in addiction

The nucleus accumbens (NAc) is a structure within the ventral striatum and a critical component of dopaminergic mesocorticolimbic circuitry governing reward-related learning, motivation, and cognition. The NAc receives dense dopaminergic projections from the midbrain in addition to extensive glutamatergic innervations from cortical, limbic, and thalamic areas. GABAergic medium spiny neurons (MSNs), which comprise over 95% of the total neuronal population in the NAc, project to structures mediating motor behavior. Thus, the NAc acts as an interface integrating cortical and limbic information and is thus critically positioned to regulate motivated behavior and learning (Groenewegen et al., 1999). MSNs within the NAc can be further distinguished by the expression of the dopamine receptor 1 subtype (D1-MSN) or receptor 2 subtype (D2-MSN), with a small percentage expressing both types. These distinct MSN subpopulations have divergent projection targets and have distinct roles in addiction-related behavior (Lobo and Nestler, 2011).

The NAc is composed of two distinct subdivisions: core and shell. Each of these regions has been shown to possess distinct functions, circuitry, and signaling mechanisms (Voorn et al., 2004). The core region is well-connected to motor circuitry and is generally thought to play a major part in initializing reward-related motor activities, controlling the acquisition of drug-seeking behavior. The shell is more heavily innervated by cortical and limbic regions, and is thus essentially involved in motivation and reward-related processes, including motivation to seek drugs.

Extensive experimental evidence indicates that the NAc is a crucial locus for drug-induced plasticity and mediates many complex behavioral and motivational aspects of addiction (Kauer and Malenka, 2007; Lüscher and Malenka, 2011). While augmented dopaminergic release from the ventral tegmental area (VTA) induced by drugs of abuse drives early behavioral responses to addicting drugs (Kauer and Malenka, 2007), alterations in NAc glutamatergic synaptic transmission that develop as a consequence of repeated, long-term drug experience have been well-demonstrated to underlie the persistence of drug-seeking behavior (Wolf, 2016), likely representing the strength of reward-related associative learning about drug experiences. In preclinical studies of addiction, activity and plasticity at glutamatergic afferents on NAc MSNs critically regulate the rewarding and reinforcing effects of drugs, rendering the NAc a prime target for investigating how experience with drugs of abuse alters brain function and drives persistent vulnerability to relapse.

Animal behavioral models of drug addiction

Behavioral sensitization

Repeated administration of a psychostimulant drug produces progressive increases in the locomotor-activating properties of the drug, a phenomenon known as behavioral sensitization. Produced by repeated experimenter-administered drug injections, the development of sensitization is associated with a number of behavioral and neurobiological adaptations within mesocorticolimbic dopamine circuitry implicated in addiction (Vanderschuren and Pierce, 2010), and therefore may be used as an index of

drug-related adaptations in reward circuit function. Sensitized animals acquire drug self-administration more easily (Piazza et al., 1989) and work harder under progressive ratio schedules of reinforcement to obtain drug (Lorrain et al., 2000; Mendrek et al., 1998), demonstrating that mechanisms of plasticity underlying behavioral sensitization might overlap with motivational processes in the brain that mediate drug-seeking behavior. Furthermore, sensitization is a long-lasting behavioral adaptation (Paulson et al., 1991), suggesting that neurobiological alterations underlying this phenomenon might play a role in persistent vulnerability to relapse.

Self-administration models

Drug self-administration procedures are dependent ("contingent") on an animal's response, providing a useful model for assessing the reinforcing and motivational properties of a drug (Shaham et al., 2003). To model human conditions in which drug-taking is periodically terminated due to the negative consequences of addiction, animals may be placed in forced abstinence following the acquisition of drug self-administration. Alternatively, extinction training is used to extinguish behavioral responding to the availability of a drug. The renewal, or reinstatement, of drug seeking behavior may be elicited by presenting the animal with cues associated with previous drug use, a stressor, or drug re-exposure—potent triggers that precipitate relapse in humans. Thus, self-administration models provide a relevant means of studying drug-induced plasticity associated with relapse to drug-seeking behavior.

Glutamatergic synaptic plasticity in cocaine addiction: role of AMPA receptors

In the absence of glutamatergic input, the MSN membrane potential naturally resides in a hyperpolarized state (-80 mV), rendering these cells normally quiescent. However, strong coincident glutamatergic input from afferents impinging on MSNs can cause a transition from low to high activity due to activation of post-synaptic glutamate receptors that depolarize neurons to spike thresholds (Surmeier et al., 2007). Striatal circuits therefore rely on strong glutamatergic afferent input to drive their activity. Glutamate inputs activate MSNs in the NAc predominantly by acting at ionotropic AMPA-type glutamate receptors (AMPARs) (Hu and White, 1996; Pennartz et al., 1990), which mediate the majority of excitatory synaptic transmission in the brain. Structurally, AMPARs are tetramers consisting of GluA1-4 subunits typically organized as dimers of dimers. Within the NAc, the majority of AMPARs consist of GluA1/2, with a smaller population lacking the GluA2 subunit and therefore existing as either homomeric GluA1 or GluA1/3 (Reimers et al., 2011; Wenthold et al., 1996). GluA2-lacking receptors allow higher channel conductance, are permeable to calcium, and display inward rectification due to voltage-dependent modulation by intracellular polyamines, thus having robust effects on synaptic plasticity that are distinct from GluA2-containing receptor compositions (Thiagarajan et al., 2007).

Multiple lines of evidence indicate that experience-dependent alterations in glutamate transmission in the NAc, such as those produced by repeated exposure to cocaine, involve alterations in post-synaptic signaling mediated by AMPA-type receptors (Pierce and Wolf, 2013; Wolf and Ferrario, 2010). Early studies found that AMPAR

activation in the NAc is necessary for drug seeking in a number of animal models (Conrad et al., 2008; Cornish et al., 1999; Suto et al., 2004). Upregulation of AMPAR signaling at excitatory synapses on NAc MSNs may strengthen drug-related associations over long periods of time and drive robust behavioral responding to drug re-exposure or cues associated with drugs. Consistent with this, repeated cocaine exposure produces a progressive increase in MSN AMPAR signaling during abstinence that heightens the sensitivity of MSNs to glutamatergic input, and is a key factor in the development and persistence of addiction-related behavior such as behavioral sensitization, craving, and relapse (Wolf and Ferrario, 2010). Cell surface expression of GluA1 AMPAR subunits is increased in the NAc during mid-late abstinence (Day 7-45), but is not present immediately after the last cocaine exposure (Day 1) (Boudreau and Wolf, 2005; Boudreau et al., 2007; Conrad et al., 2008; Ferrario et al., 2011; Ghasemzadeh et al., 2009), indicating that accumulation of synaptic AMPARs following cocaine treatment requires time to develop. Additionally, electrophysiology studies reveal that AMPAR signaling is potentiated in the NAc shell in late abstinence (Day 10-45), again with no effect observed in early abstinence (Conrad et al., 2008; Kourrich and Thomas, 2009; Pascoli et al., 2014, 2011; Terrier et al., 2016).

In contrast, during abstinence from cocaine, exposure to a cocaine challenge injection reverses, or "depotentiates," cocaine-dependent increases in NAc shell AMPAR signaling, promoting a reduction in glutamate sensitivity of MSNs. Within 24 hours following cocaine challenge, cell surface expression of GluA1 and GluA2 is reduced (Boudreau et al., 2007; Ferrario et al., 2009), and excitatory synaptic currents are

attenuated (Kourrich et al., 2007). Furthermore, exposure to stress is similarly capable of inducing synaptic depotentiation in NAc MSNs (Rothwell et al., 2011), demonstrating that both drug re-exposure and stress, common triggers for evoking relapse, promote similar reductions in NAc excitatory synaptic strength. Synaptic adaptations associated with relapse-inducing stimuli could provide a neurobiological correlate for understanding the processes that underlie vulnerability to relapse. Alterations in AMPAR-mediated synaptic strength in the NAc may therefore drive renewed drug-seeking behavior during withdrawal. However, the mechanisms underlying bidirectional alterations in AMPAR synaptic transmission in the NAc have not been well defined.

One possibility is that re-exposure to cocaine or other relapse-associated stimuli promotes removal of AMPARs from the membrane, yielding a reduction in synaptic strength. Indeed, a common mechanism throughout the brain for altering glutamatergic synaptic strength involves regulating the trafficking of AMPARs into and out of the synapse on a rapid timescale (Derkach et al., 2007; Shepherd and Bear, 2011). Several lines of evidence indicate that AMPAR trafficking mechanisms play a role in cocaine-induced alterations in glutamatergic synaptic strength in the NAc. Several studies found that cell surface expression of GluA1 and GluA2 AMPAR subunits is decreased 24 hours after a cocaine challenge (Boudreau et al., 2007; Ferrario et al., 2011), indicating that AMPARs are internalized following cocaine re-exposure. Additionally, blocking endocytosis of AMPARs with a synthetic peptide prevents the expression of amphetamine sensitization and the induction of long-term depression at NAc MSN synapses (Brebner et al., 2005), demonstrating a critical role for AMPAR trafficking in

drug-related behavior and synaptic plasticity. Regulation of AMPAR trafficking mechanisms may therefore mediate the reversal of potentiated AMPAR signaling induced by repeated cocaine, suggesting a process through which synapses rapidly adapt to drug re-exposure.

Ex vivo cocaine: a model for understanding drug re-exposure-induced plasticity

Understanding the detailed cellular and temporal mechanisms of cocaine-induced synaptic plasticity requires isolating specific candidate signaling mechanisms. To investigate the specific mechanisms of AMPAR synaptic depotentiation in the NAc induced by cocaine re-exposure, we developed an *ex vivo* drug challenge model that allows us to pharmacologically target and manipulate specific signaling factors necessary for cocaine-induced plasticity. This approach could allow for resolving specific mechanisms as well as temporal parameters of NAc synaptic plasticity—fine details that are difficult to assess in *in vivo* models. The goal of this model is to provide a screening tool to identify candidate mechanisms of plasticity that might guide future investigations of drug-related synaptic plasticity.

In this model, animals undergo repeated injections of cocaine that produce robust behavioral sensitization. Identical to *in vivo* sensitization studies from our lab (Kourrich et al., 2007), animals are then placed in abstinence for 10-14 days. Following the abstinence period, however, instead of an *in vivo* challenge injection, the cocaine re-exposure is carried out by perfusing slices from pre-treated animals in an artificial cerebrospinal fluid (ACSF) solution containing 10 μM cocaine. This concentration of

cocaine was chosen since it has been shown to promote plasticity in acute slice preparations without causing local anesthetic effects (Brodie and Dunwiddie, 1990; Schilström et al., 2006). Slices are exposed to *ex vivo* cocaine for a discrete time period (10 min), modeling *in vivo* cocaine re-exposure.

In a recent study (Jedynak et al., 2016), we sought to validate this model by testing whether *ex vivo* cocaine produces bidirectional AMPAR-mediated plasticity in the NAc, similar to plasticity our lab has observed following *in vivo* (Kourrich et al., 2007). Mirroring *in vivo* cocaine drug re-exposure, incubation of acute slices in cocaine reversed the potentiation of AMPAR-mediated synaptic transmission in the NAc shell and core, demonstrating that *ex vivo* cocaine can bidirectionally modulate excitatory synaptic transmission in the NAc. We additionally found that *ex vivo* amphetamine is similarly capable of depotentiating enhanced AMPAR transmission during abstinence, again reflecting *in vivo* amphetamine data from our lab (Jedynak et al. 2016). Interestingly, we furthermore observed that the region-specific effects (i.e. NAc core vs. shell) of *in vivo* cocaine and amphetamine on AMPAR plasticity once again held true in the *ex vivo* model, with cocaine exerting effects in both shell and core while amphetamine preferentially altered AMPAR transmission in the shell only. Given these striking similarities between the ability of *in vivo* and *ex vivo* psychostimulant exposure to bidirectionally alter glutamatergic transmission in the NAc, this model appears to be a highly viable and useful approach for probing cellular signaling mechanisms of synaptic plasticity.

Purpose

In summary, AMPA receptors mediate critical alterations in glutamatergic synaptic plasticity in the NAc evoked by cocaine. Our lab and others have observed that while AMPAR signaling is potentiated during abstinence from cocaine, a single re-exposure to cocaine or cues associated with drug use is sufficient to reverse this plasticity, inducing synaptic depotentiation. This synaptic adaptation elicited by common triggers for relapse in humans might represent a neurobiological correlate for studying relapse vulnerability in the brain. However, the cellular mechanisms underpinning this plasticity have not been characterized.

In the following chapters, my purpose is to begin to characterize cellular signaling mechanisms important for cocaine-induced bidirectional plasticity in the NAc. Drawing from an expanding literature on endogenous mechanisms of plasticity commonly engaged by drugs of abuse, I describe the roles of a number of signaling mechanisms in cocaine-evoked bidirectional plasticity:

1. Characterize the role of the mGluR5 receptor and protein synthesis in bidirectional AMPAR plasticity in the NAc, comparing core and shell regions.
2. Resolve the temporal dynamics of cocaine-induced plasticity, including onset and duration of AMPAR plasticity.
3. Examine the role of the neuromodulators dopamine and endocannabinoids in NAc glutamatergic plasticity.
4. Characterize cocaine-induced synaptic depotentiation involving specific dopamine receptor sub-types on specific MSN cell populations.

Chapter 1: Activation of mGluR5 and protein synthesis in the NAc are necessary for AMPAR depotentiation evoked by cocaine re-exposure

Introduction

Group I mGluRs: role in striatal synaptic plasticity

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors (GPCRs) highly expressed at excitatory synapses throughout the brain that critically modulate glutamatergic synaptic activity and plasticity. Group I mGluRs comprise mGluR1 and mGluR5, G_q-coupled receptors that promote cleavage of the membrane phospholipid PIP2 through activation of PLC, generating DAG and IP3 that mobilize internal calcium stores, increasing the post-synaptic intercellular concentration of calcium. Augmented calcium levels activate a host of downstream cellular effectors and targets that modify glutamatergic synaptic transmission. Located outside of the active zone within peri-synaptic regions (Luján et al., 1996), mGluR1/5 are well positioned to modulate the activity of nearby ionotropic receptors.

In the striatum, glutamatergic synapses on MSNs are capable of expressing synaptic depression through several mechanisms. An extensive body of evidence has demonstrated that group I mGluRs mediate a prominent form of striatal long-term depression (mGluR-LTD) at excitatory synapses (Lüscher and Huber, 2010). Classic studies characterizing mGluR-LTD in the striatum identified that pharmacological activation of mGluR1/5 or high-frequency stimulation (HFS) is sufficient to induce synaptic depression at MSN synapses (Bellone et al., 2008; Gladding et al., 2009; Gubellini et al., 2004). Activation of post-synaptic mGluR1/5 initiates a cascade of

intercellular signaling mechanisms that participate in reducing excitatory synaptic strength through a number of pathways. In the nucleus accumbens (NAc), mGluR1/5 activation promotes a long-lasting reduction in glutamate release probability (Robbe et al., 2002) coupled with internalization of AMPARs from the post-synaptic membrane (Mangiavacchi and Wolf, 2004; McCutcheon et al., 2011). NAc mGluR1/5 signaling thus regulates the activity of striatal circuits by dampening excitatory input to MSNs while reducing the responsiveness of neurons to glutamate.

Group I mGluRs in cocaine-seeking behavior and plasticity

Group I mGluR receptors have been demonstrated to critically mediate addiction-related behavior. In particular, the role of mGluR5 in cocaine-seeking behavior and synaptic plasticity in the NAc has been well-characterized. Transgenic mice lacking mGluR5 receptors do not develop behavioral sensitization to cocaine, and furthermore do not acquire cocaine self-administration behavior (Chiamulera et al., 2001). Intra-NAc administration of an mGluR5 antagonist attenuates cocaine-primed and cue-primed reinstatement of cocaine seeking behavior in the NAc core (Wang et al., 2013) and shell (Kumaresan et al., 2009). Consistent with this, stimulation of mGluR5 receptors in the NAc core (Wang et al., 2013) and shell (Schmidt et al., 2013) promotes cue- and cocaine-primed reinstatement of seeking behavior. These findings indicate that mGluR5 signaling in the NAc shell and core plays a crucial role in cocaine-seeking behavior. However, the contribution of mGluR5 to cocaine-induced synaptic plasticity in the NAc has not been fully defined. Given the known role of group I mGluRs in regulating excitatory synaptic

transmission in the NAc (Robbe et al., 2002), activation of NAc mGluR5 following abstinence or extinction from cocaine might promote dampening of synapses potentiated by previous cocaine experience, representing a potential strategy for reducing cocaine craving and seeking behavior. Consistent with this, one study found that activation of group I mGluRs in the NAc following prolonged abstinence (>45 d) from cocaine self-administration eliminates high-conductance calcium-permeable AMPARs from the synapse, reducing glutamatergic transmission (McCutcheon et al., 2011). Previous studies have indicated that a reduction in elevated AMPAR synaptic transmission following cocaine exposure requires mGluR5 signaling mechanisms that promote phosphorylation and rapid endocytosis of AMPARs (Collingridge et al., 2004). For example, one report found that in addition to mGluR5, cocaine-primed reinstatement of seeking requires activation of PLCγ and PICK1, two cellular effectors activated downstream of mGluR5 that cooperate to initiate endocytosis of AMPARs (Schmidt et al., 2013). Given that re-exposure to cocaine enhances glutamatergic tone in the NAc (Kalivas, 2009; Knackstedt and Kalivas, 2009), subsequent engagement of mGluR5s on MSNs could function to dampen potentiated glutamatergic synaptic transmission through mGluR5-mediated internalization of AMPARs.

Facilitation of synaptic plasticity by protein synthesis

A common requirement for mGluR1/5-mediated plasticity throughout the brain involves rapid protein synthesis (Lüscher and Huber, 2010). Stimulation of mGluR1/5 promotes local synthesis of several LTD-associated proteins that participate in regulating

AMPAR trafficking and endocytosis at the synapse (Huber et al., 2000). For example, induction of mGluR5-LTD and AMPAR endocytosis requires rapid dendritic synthesis of Arc protein, which functions to couple changes in neuronal activity to rapid synaptic plasticity by regulating AMPAR endocytosis and inducing synaptic depression (Chowdhury et al., 2006; Shepherd and Bear, 2011; Waung et al., 2008). mGluR1/5 activation therefore stimulates a number of proteins including Arc that negatively regulate AMPAR cell surface expression. Importantly, synthesis of these proteins is engaged by changes in neuronal activity associated with addiction-related behavior: a mechanism requiring rapid protein synthesis is required for sustained calcium-permeable AMPAR transmission in the NAc during incubation of cocaine craving (Scheyer et al., 2014), and protein synthesis is similarly necessary for cue-evoked reinstatement of cocaine seeking (Smith et al., 2014). Thus, rapid protein synthesis in the NAc may be an important factor mediating depotentiation of enhanced AMPAR transmission during abstinence from cocaine.

The purpose of this study was to investigate the role of mGluR5 and protein synthesis in cocaine-induced depotentiation of AMPAR synaptic strength in the NAc. Furthermore, we examined the region-specificity of this plasticity by comparing mechanisms in both the NAc shell and core.

Materials and methods

Animals

Adult male (P49-70) C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine, USA) were used. All animals were group-housed in a temperature- and humidity-controlled environment on a 12 hr light/12 hr dark cycle, with food and water available *ad libitum.* All experiments were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Behavioral sensitization

Prior to testing, mice were habituated to experimenter handling, *i.p*. injections, and the testing environment (individual activity boxes, 8.5 x 17.5 x 9 in) over two days. On five consecutive testing days, mice were habituated to the testing environment for 30 min before receiving an intraperitoneal injection of either cocaine (15 mg/kg) or saline. Mice were immediately placed back into the testing environment, and their activity was monitored for 90 min using a video-based tracking system (Any-Maze, Stoelting, WI, USA). Animals were returned to their home cages at the end of each testing period. Following the last day of testing, animals remained in their home cages in the colony for 10 – 14 days before electrophysiology experiments were performed. To determine whether repeated cocaine treatment produced behavioral sensitization, the locomotor activity (distance traveled) during the first 30 minutes of the session on Day 1 and Day 5 was compared, pooling all cocaine-treated animals. A t-test was used to confirm a significant difference in locomotor activity between Day 1 and Day 5, indicating the development of behavioral sensitization to cocaine.

Ex vivo drug application

Drugs were prepared in a stock solution and added at the final concentration to the ACSF bath solution. A between-cell experimental design was utilized in which slices were first placed in a separate chamber containing the drug or control ACSF prior to recording synaptic activity. For these *ex vivo* experiments, slices were exposed to one of the following conditions:

1) no challenge (exposure to ACSF only)

2) *ex vivo* cocaine challenge (10 µM; 10 min)

3) *ex vivo* MTEP (specific mGluR5 antagonist; 5 µM; 10 min) or cycloheximide (protein synthesis inhibitor; 60 µM; 30 min) alone.

4) *ex vivo* MTEP *or* cycloheximide (10 min) followed by 10 µM cocaine + antagonist (10 min).

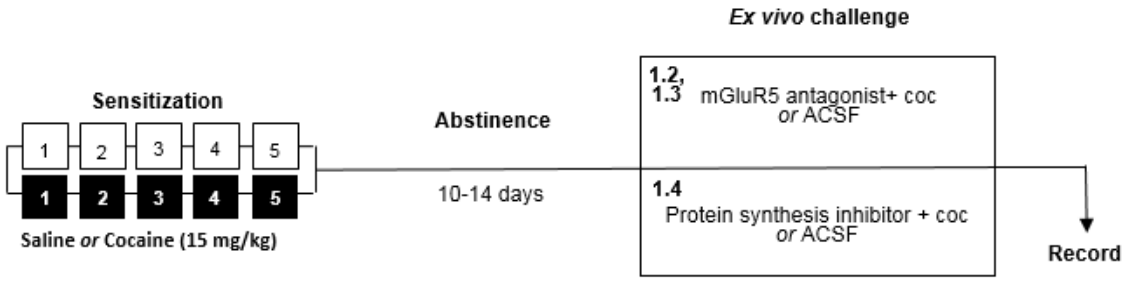

Figure 1.1. Timeline of experimental manipulations. Mice received five once-daily injections of cocaine or saline, followed by 10-14 days of abstinence in their home cages. Brain slices were prepared following the abstinence period. To examine the role of mGluR5 and protein synthesis, the mGluR5 inhibitor MTEP (5 µM) *or* the protein synthesis inhibitor cyclohexamide (60 µM) was applied prior to and during *ex vivo* cocaine. Recording took place immediately following drug conditions.

Following the timed drug exposure, slices were transferred to the recording chamber where standard ACSF continuously perfused the slices. Recordings took place up to 1 hour following *ex vivo* drug exposure.

Slice electrophysiology

Following 10–14 days of abstinence from cocaine treatment, mice were anesthetized with isoflurane, decapitated, and the brain rapidly removed. Parasagittal slices (250 µm) containing the nucleus accumbens shell and core were prepared in ice-cold artificial cerebrospinal fluid (ACSF) saturated with 95% O_2 / 5% CO_2 and containing (in mM): 6 kynurenic acid, 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, 1.3 $MgSO_4$, and 2.5 $CaCl_2$. Slices rested for at least 30 min in a holding chamber at room temperature containing standard ACSF continuously saturated with 95% O_2 / 5% CO_2 and composed of (in mM): 119 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, 1.3 $MgSO_4$, and 2.5 $CaCl_2$. During recording, picrotoxin (100 µM) and lidocaine (0.7 µM) were included to block $GABA_A$-mediated inhibitory transmission and action potentials, respectively. Neurons in the NAc shell were visualized on an upright microscope using infrared differential interference contrast (IR-DIC) optics. MSNs were identified by their morphology and hyperpolarized resting membrane potential (-70 to -80 mV). To assess excitatory synaptic transmission mediated by AMPARs, cells were voltage-clamped at -80 mV using an Axon Instruments MultiClamp 700A (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes (3-5 MΩ) were filled with a cesium-gluconate-based internal solution containing (in mM): 117 cesium

gluconate, 2.8 NaCl, 20 HEPES, 0.4 EGTA, 5 tetraethylammonium-Cl, 2 MgATP, and 0.3 MgGTP (pH 7.2-7.4). Data were filtered at 2 kHz, digitized at 5 kHz, and collected using custom Igor Pro software (Wavemetrics, Lake Oswego, OR, USA). A depolarizing step (4 mV, 100 ms) was generated at the beginning of each sweep to monitor series resistance (10-40 MΩ) and input resistance (>500 MΩ). MiniAnalysis software (Synaptosoft, Decatur, GA, USA) was used offline to analyze mEPSC amplitude and frequency.

2.5 Drugs

Cyclohexamide and MTEP were purchased from Tocris Bioscience (Bristol, United Kingdom). Lidocaine and picrotoxin were purchased from Sigma Aldrich (St. Louis, MO, USA). Cocaine was obtained from Boynton Health Services Pharmacy (University of Minnesota, Minneapolis, MN, USA). Drugs were prepared in a stock solution at 100 – 1000 times the desired concentration and added into standard ACSF at the final concentration.

2.5 Statistical analysis

All data are presented as group mean ± SEM. Statistical significance was assessed with a Student's t-test or one-way ANOVA using JMP Pro (SAS, Cary, NC, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Tukey HSD *post hoc* tests were used for pairwise comparisons where appropriate. The threshold for significance was $p<0.05$.

Results

In the striatum, group I mGluRs prominently mediate the induction of synaptic depression at excitatory synapses on MSNs (Lüscher and Huber, 2010). In the NAc, activation of post-synaptic group I mGluRs has been shown to promote reduced presynaptic glutamate release probability and increased trafficking of AMPARs (McCutcheon et al., 2011; Robbe et al., 2002). Thus, we investigated whether blockade of mGluR5-dependent signaling with *ex vivo* application of the antagonist MTEP prevents the cocaine-induced depotentiation of AMPAR-mediated signaling in the NAc shell and core.

Activation of mGluR5 in the NAc shell but not core is necessary for cocaine-induced synaptic depotentiation.

Mice were treated with five once-daily injections of saline or cocaine (15 mg/kg *i.p.*) that produced robust behavioral sensitization (137.1 ± 9.865 mean difference in meters travelled/30 min between injection day 1 and day 5; $t_{(1,22)} = 13.9$, $p<0.0001$). Following 10-14 days of abstinence, animals were sacrificed and acute slices containing the NAc shell were prepared. As demonstrated in previous experiments from our lab (Jedynak et al., 2016), cocaine pre-treatment increased the amplitude (measured in picoamperes of current, *pA*) of mEPSCs, a direct measure of AMPAR function, in shell MSNs, which was subsequently reversed by an *ex vivo* cocaine bath challenge (**figure 1.2B**-left; Sal (10.87 ± 0.46, 8 cells/4 animals), Coc (14.62 ± 0.90, 11 cells/5 animals), Coc–Coc (10.52 ± 0.32, 9 cells/5 animals); $F_{(6,71)}=5.93$, $p<0.001$). However, incubation

of acute slices in MTEP (5 μM) prior to cocaine challenge prevented the cocaine-induced reductions in mEPSC amplitude (**figure 1.2B**-left; Coc–MTEP–Coc (14.05 ± 0.49, 8 cells/3 animals), $p<0.001$). Interestingly, MTEP pre-treatment did not prevent the cocaine challenge-induced reversal of AMPAR mEPSC frequency (events per second, *Hz*) [**figure 1.2B**-right; Sal (3.95 ± 0.42), Coc (5.78 ± 0.61), Coc–Coc (3.33 ± 0.36), Coc–MTEP–Coc (3.87 ± 0.50); $F_{(6,66)}=3.90$, $p=0.002$]. Additionally, in slices from cocaine-treated animals, while bath application of MTEP alone had no effect on baseline mEPSC amplitudes (Coc–MTEP: 13.80 ± 0.52) or frequency (Coc–MTEP: 6.27 ± 1.11) (data not shown), MTEP application to slices from saline-treated animals (Sal–MTEP) significantly increased mEPSC amplitude (14.20 ± 0.97) and trended toward elevating frequency (6.21 ± 1.02, $p=0.089$) (data not shown) compared with saline-no challenge controls.

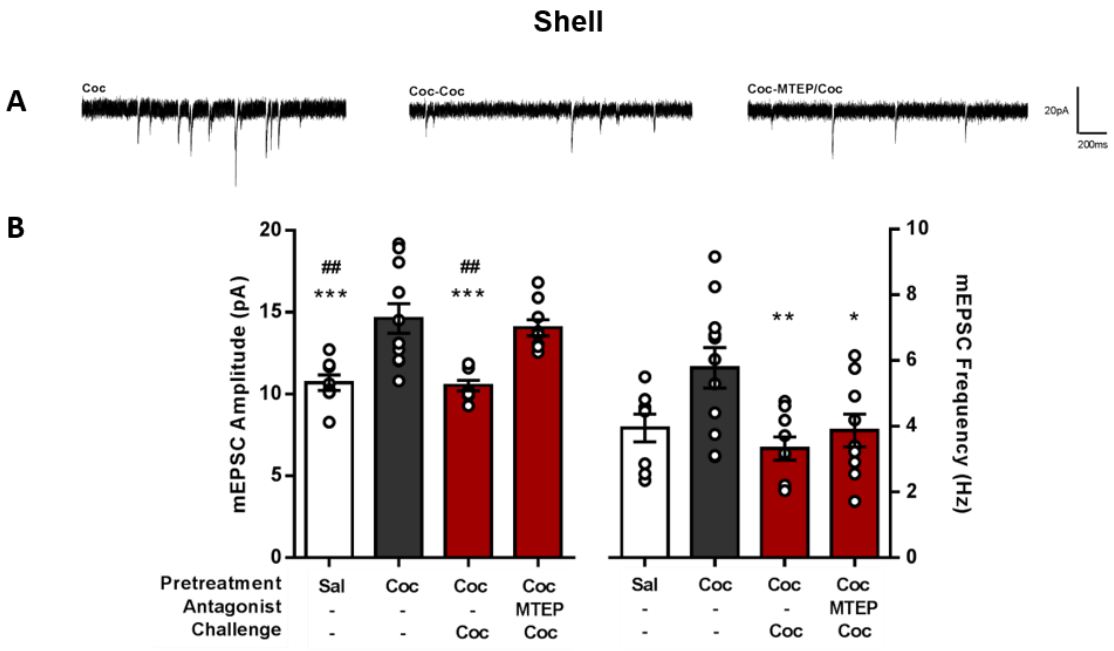

Figure 1.2. Activation of mGluR5 in the NAc shell is necessary for cocaine-induced synaptic depotentiation. (A) Representative mEPSC current traces from NAc shell neurons. (B) Mean mEPSC amplitude and frequency in the NAc shell from saline (Sal), cocaine + no challenge (Coc), cocaine + cocaine challenge (Coc-coc), cocaine + MTEP/cocaine (Coc-MTEP-Coc). All data are presented as mean ± SEM. *p<0.05 vs. Coc, ** p<0.01 vs. Coc, *** p<0.001 vs Coc; # p<0.05 vs. Coc-MTEP-Coc, ## p<0.01 vs. Coc-MTEP-Coc.

In the NAc core, cocaine significantly increased mEPSC amplitude compared with saline controls [**figure 1.3B**-left; Sal (11.41 ± 0.48, 9 cells/4 animals), Coc (14.20 ± 1.08, 8 cells/4 animals), Coc–Coc (11.30 ± 0.43, 14 cells/5 animals); $F_{(5,57)}$=4.57, p=0.002]; however, unlike the shell, incubation of acute slices in MTEP prior to cocaine bath challenge failed to prevent the reduction in mEPSC amplitude [**figure 1.3B**-left; Coc–MTEP–Coc (11.85 ± 0.97, 11 cells/3 animals), p<0.002]. MTEP application alone had no effect on mEPSC amplitude in slices from saline- (Sal–MTEP: 14.20 ± 1.08) or cocaine-treated mice (Coc–MTEP: 14.51 ± 1.04) (data not shown) compared with their respective controls. No significant effects were observed on mEPSC frequency in the

NAc core (**figure 1.3B**-right; $F_{(5,57)}$=2.04, p=0.09). Thus, while the phenomena of cocaine-induced depotentiation in the NAc core versus shell are outwardly similar, the mechanisms appear to be different, with NAc shell depotentiation relying on mGluR5 activation.

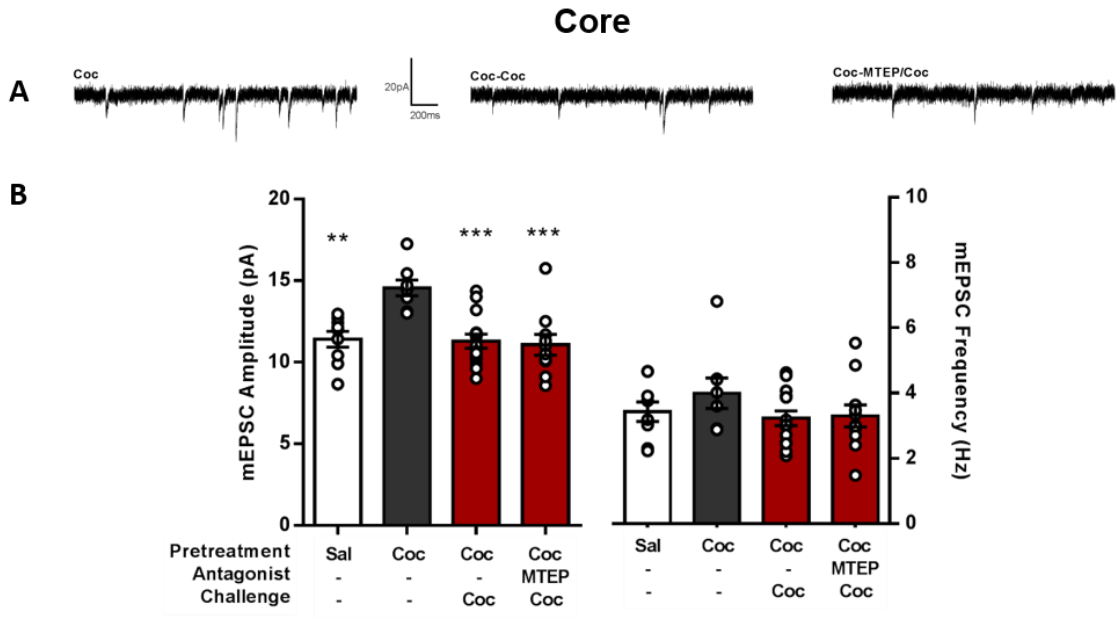

Figure 1.3. Synaptic depotentiation in the NAc core does not require mGluR5 activity. (A) Representative mEPSC current traces from NAc core neurons. (B) Mean mEPSC amplitude and frequency in the NAc core from saline (Sal), cocaine + no challenge (Coc), cocaine + cocaine challenge (Coc-coc), cocaine + MTEP/cocaine (Coc-MTEP-Coc). All data are presented as mean ± SEM. ** p<0.01 vs. Coc, *** p<0.001 vs Coc.

Protein synthesis mediates depotentiation of AMPAR signaling in the NAc shell

A common mechanism for mGluR-dependent long-term depression in many brain regions, including the striatum, is the reliance on rapid protein synthesis (Lüscher and Huber, 2010; Yin and Lovinger, 2006). Since we observed that mGluR5 signaling selectively mediates depotentiation of AMPAR synaptic strength in the NAc shell, we

chose to focus this investigation on the NAc shell, excluding the core. Consistent with an mGluR5 mechanism, we found that incubation of slices from cocaine-treated mice in the protein translation inhibitor cyclohexamide (60 μM) for 30 min prior to cocaine bath challenge also prevented reductions in mEPSC amplitude [**figure 1.4B**-left; Sal (10.87 ± 0.46, 8 cells/4 animals), Coc (14.62 ± 0.90, 11 cells/5 animals), Coc–Coc (10.52 ± 0.32, 9 cells/5 animals); Coc–Cyclo–Coc (13.45 ± 0.60, 14 cells/6 animals), $F_{(6,66)}$ =5.93, p<0.001]. However, similar to the MTEP findings, cycloheximide pre-treatment did not prevent the cocaine challenge-induced depotentiation of AMPAR mEPSC frequency [**figure 1.4B**-right; Sal (3.95 ± 0.42), Coc (5.78 ± 0.61), Coc–Coc (3.33 ± 0.36); Coc–Cyclo–Coc (3.52 ± 0.56); $F_{(6,66)}$=3.90, p=0.002]. These findings confirm that depotentiation of synaptic strength in the NAc shell during abstinence from cocaine requires rapid protein synthesis, suggesting selective engagement of mGluR5 and protein synthesis mechanisms in the shell.

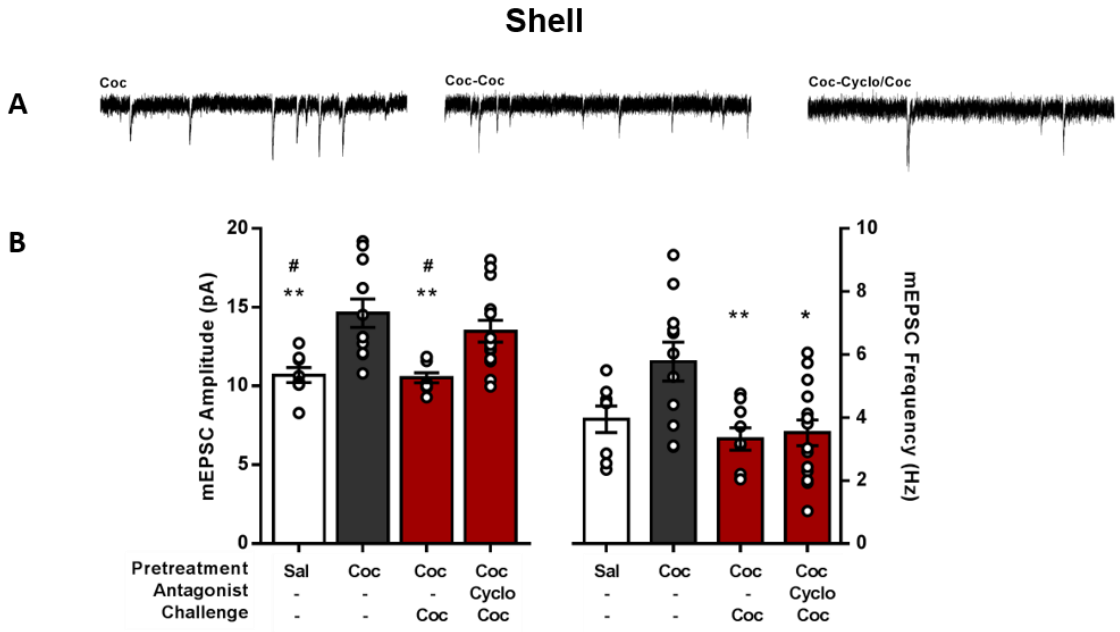

Figure 1.4. NAc shell synaptic depotentiation requires protein synthesis. (A) Representative mEPSC current traces from NAc shell neurons. (B) Mean mEPSC amplitude and frequency in the NAc shell from saline (Sal), cocaine + no challenge (Coc), cocaine + cocaine challenge (Coc-coc), cocaine + Cyclo/cocaine (Coc-Cyclo-Coc). All data are presented as mean ± SEM. *p<0.05 vs. Coc, ** p<0.01 vs. Coc; # p<0.05 vs. Coc-Cyclo-Coc.

Discussion

Group I mGluRs are widely expressed throughout the brain, comprising a dominant mechanism for modulating synaptic efficacy and plasticity at glutamatergic synapses. In the striatum, mGluR1/5 signaling has been demonstrated to critically regulate circuits that influence motivated learning and behavior, as well as reward and reinforcement (Olive, 2010; Philibin et al., 2011). Expanding on previous studies investigating the role of mGluR5 in cocaine-seeking behavior (Kumaresan et al., 2009; Schmidt et al., 2013), we found here that depotentiation of enhanced AMPAR synaptic transmission in the NAc requires mGluR5 signaling, indicating that cocaine re-exposure

during abstinence engages mGluR5 signaling mechanisms that promote a reduction in AMPAR-mediated synaptic strength.

Following protracted abstinence or extinction from repeated cocaine exposure, renewed exposure to cocaine transiently increases glutamate release in the NAc (McFarland et al., 2003; Pierce et al., 1996; Reid and Berger, 1996), an effect that has been demonstrated to be a causal factor driving reinstatement behavior (Baker et al., 2003). Augmentation of glutamatergic transmission elicited by drug re-exposure indicates a means by which mGluR5 may be engaged by glutamatergic signaling in the NAc. mGluR5s are densely expressed in the NAc (Lu et al. 1999), where they provide a prominent mechanism for reducing synaptic transmission at excitatory synapses. Activation of these receptors may therefore provide a mechanism for decreasing the responsiveness of MSNs to glutamatergic input, dampening potentiated excitatory synaptic transmission.

Throughout the brain, mGluR1/5 couples to intercellular mechanisms that promote synaptic depression through endocytosis of AMPARs. Indeed, studies in the hippocampus (Gladding et al., 2009; Snyder et al., 2001) and bed nucleus of the stria terminalis (Grueter et al., 2008) have repeatedly demonstrated that mGluR1/5-mediated LTD is post-synaptically maintained through AMPAR endocytosis. In the NAc, while mGluR1/5-LTD involves a significant pre-synaptic contribution due to mGluR1/5-mediated mobilization of endogenous cannabinoids that negatively regulate glutamate release probability (Robbe et al., 2002), evidence indicates that mGluR-1/5 synaptic depression is also expressed through post-synaptic endocytosis of AMPARs

(Mangiavacchi and Wolf, 2004a; McCutcheon et al., 2011). In the NAc, mGluR1/5 signaling is therefore capable of both reducing the strength of excitatory drive as well as the post-synaptic sensitivity of NAc MSNs to glutamatergic input.

This rapid dampening of glutamatergic synaptic strength may be a critical factor in triggering a renewal of cocaine seeking behavior, given that activation of NAc mGluR5 during abstinence or extinction is sufficient to induce cocaine-primed and cue-primed reinstatement behavior (Schmidt et al., 2013; Wang et al., 2013). Our data here demonstrates that during abstinence from repeated cocaine, mGluR5 signaling in the NAc is necessary for the reversal of potentiated AMPAR signaling induced by cocaine re-exposure *ex vivo*, preventing the induction of synaptic depotentiation normally produced by cocaine.

However, these findings contrast with other evidence suggesting that mGluR1/5-mediated synaptic depression in the NAc is impaired following chronic cocaine exposure. During abstinence from repeated cocaine, the ability of the mGluR1/5 agonist DHPG to induce synaptic depression at NAc MSN synapses is impaired (Huang et al., 2011), an effect that is not dependent on MSN cell sub-type (Huang et al., 2015). This could reflect a downregulation of mGluR1/5 total expression (Huang et al., 2011) or cell surface levels observed by a number of studies following chronic cocaine exposure (Knackstedt et al., 2010; Swanson et al., 2001). Moreover, impairment of mGluR1/5-LTD has been suggested to be region-specific, as different groups have observed impaired mGluR1/5 plasticity in the NAc shell (Huang et al., 2011) or core (Swanson et al., 2001) during protracted abstinence from cocaine. Indeed, we observed that application of the mGluR5

antagonist MTEP was effective at preventing the cocaine-induced depotentiation selectively in the NAc shell, without blocking the induction of plasticity in the NAc core. This could reflect a specific loss of mGluR5-mediated efficacy at NAc core synapses due to decreased receptor availability; however, given the limited observations of this effect, more research is needed to characterize alterations in mGluR5 expression levels, presence on the cell surface, or coupling with intercellular signaling mechanisms that mediate a reduction in synaptic transmission.

Additionally, many of the studies cited above did not differentiate between mGluR1 and mGluR5 pharmacologically, using the group I mGluR agonist DHPG to induce mGluR1/5-LTD in the NAc. Given that these receptor sub-types have been shown to mediate divergent effects on NAc synaptic plasticity and addiction-related behavior, it is possible that the impairment of plasticity observed in the NAc is due to engagement of mechanisms outside of mGluR5 alone. Furthermore, while studies of mGluR1/5-LTD have typically characterized this form of plasticity in the NAc using electrical stimulation or pharmacological activation, these induction mechanisms may differ from the mechanisms in the present study engaged by cocaine exposure. Understanding how mGluR1/5 signaling modulates synaptic transmission will require elucidating mechanisms specific to the method of induction.

Downstream of mGluR1/5 activation, rapid protein synthesis critically maintains LTD through interactions with AMPAR endocytosis machinery (Park et al., 2008; Waung et al., 2008). A number of studies have demonstrated that mGluR1/5-mediated LTD relies on protein translation within local dendrites (Lüscher and Huber, 2010). This

rapid form of protein synthesis is activated following chronic cocaine experience: for example, a recent study revealed that protein synthesis is necessary to maintain high levels of calcium-permeable AMPARs during extended abstinence from cocaine administration (Scheyer et al., 2014). Thus, protein synthesis may assist in regulating trafficking and endocytosis of AMPARs at the synapse as a consequence of cocaine exposure. We observed that in the NAc shell, the depotentiation of AMPAR-mediated signaling requires both mGluR5 signaling and rapid protein translation (within 30 min). Future studies will be required to identify candidate proteins mediating synaptic depotentiation downstream of mGluR5 activation.

In summary, these findings define a role for mGluR5-mediated signaling in the NAc shell in mediating cocaine-induced depotentiation of AMPAR synaptic strength. We identify that mGluR5 is selectively engaged in the NAc shell, not core, during abstinence from cocaine. Furthermore, the plasticity induced by cocaine re-exposure requires rapid protein synthesis, indicating a mechanism through which mGluR5 activity promotes the rapid induction of AMPAR endocytosis.

Chapter 2: Temporal characteristics of cocaine-induced AMPAR plasticity in the NAc shell.

Introduction

Studies of drug-evoked alterations in glutamatergic synaptic function indicate dynamic and complex regulation of AMPARs in the NAc following cocaine administration. Data from our lab and others has shown that during abstinence or extinction from repeated cocaine, cell surface expression of AMPARs in the NAc is increased (Boudreau and Wolf, 2005) and AMPAR-mediated currents are enhanced (Kourrich et al., 2007; McCutcheon et al., 2011; Ortinski et al., 2012; Pascoli et al., 2011; Rothwell et al., 2011), an effect that is not present during early abstinence or following a single acute injection of cocaine (Kourrich et al., 2007). Increased AMPAR sensitivity at NAc MSNs therefore appears to be sensitive to the length of abstinence or extinction following drug exposure, requiring several days to weeks to develop. As we have previously reported (Jedynak et al., 2016), renewed cocaine exposure during abstinence depotentiates enhanced AMPAR synaptic strength, apparently resetting synapses to a basal state similar to non-drug conditions. However, the temporal parameters of this re-exposure-induced plasticity have not been well defined, including how rapidly AMPAR depotentiation is induced and whether this plasticity is transient or long-lasting.

The present study investigated the temporal parameters of NAc shell synaptic depotentiation, examining 1) the duration of AMPAR synaptic depotentiation induced by an *in vivo* cocaine challenge; and 2) the timecourse of synaptic depotentiation onset, utilizing an *ex vivo* cocaine challenge approach.

Materials and methods

Animals

Adult male (P49-70) C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine, USA) were used. All animals were group-housed in a temperature- and humidity-controlled environment on a 12 hr light/12 hr dark cycle, with food and water available *ad libitum.* All experiments were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

2.2 Behavioral sensitization

Prior to testing, mice were habituated to experimenter handling, *i.p*. injections, and the testing environment (individual activity boxes, 8.5 x 17.5 x 9 in) over two days. On five consecutive testing days, mice were habituated to the testing environment for 30 minutes (min) before receiving an intraperitoneal injection of either cocaine (15 mg/kg) or saline. Mice were immediately placed back into the testing environment, and their activity was monitored for 90 min using a video-based tracking system (Any-Maze, Stoelting, WI, USA). Animals were returned to their home cages at the end of each testing period. Following the last day of testing, animals remained in their home cages in the colony for 10 – 14 days before electrophysiology experiments were performed. For *in vivo* drug challenge experiments only, mice were handled and injected with saline during the last 2 days of the 10 – 14 day abstinence period to prevent any potential effects of stress during the challenge experiment. Mice were habituated to the testing environment for 30 min before receiving a challenge injection of cocaine (15 mg/kg) or saline,

followed by activity monitoring for 90 min. Electrophysiology experiments took place within 24 hours (h) or 5 days (d) following the challenge injection.

To determine whether repeated cocaine treatment produced behavioral sensitization, the locomotor activity (distance traveled) during the first 30 minutes of the session on Day 1 and Day 5 was compared, pooling all cocaine-treated animals. A t-test was used to confirm a significant difference in locomotor activity between Day 1 and Day 5, indicating the development of behavioral sensitization to cocaine. Animals that did not fit these criteria were excluded from recording experiments. In this and subsequent chapters, behavioral data will not be reported; see Chapter 1 for a full description.

Slice electrophysiology

Following 10–14 days of abstinence from cocaine treatment *or* either 24 hr or 5 d after an *in vivo* cocaine challenge injection, mice were anesthetized with isoflurane, decapitated, and the brain rapidly removed. Parasagittal slices (240 μm) containing the nucleus accumbens shell were prepared in 2-4°C sucrose-containing artificial cerebrospinal fluid (ACSF), after which slices rested for at least 30 min in standard ACSF at room temperature. To assess AMPAR-mediated excitatory synaptic transmission, cells were voltage-clamped at -80 mV using an Axon Instruments MultiClamp 700A (Molecular Devices, Sunnyvale, CA, USA). Data were filtered at 2 kHz, digitized at 5 kHz, and collected using custom Igor Pro software (Wavemetrics, Lake Oswego, OR, USA). MiniAnalysis software (Synaptosoft, Decatur, GA, USA) was

used offline to analyze mEPSC amplitude, frequency, rise time, and decay time. To determine the rise time and decay time, two exponentials were fit to the rise time or decay time of mEPSCs using the MiniAnalysis curve-fitting function, and the time constant was calculated between 10-90% of peak. For a more detailed description of slice preparation and recording methods, see Chapter 1.

Ex vivo drug application

For cocaine *ex vivo* experiments, cocaine was prepared in a stock solution and added at the final concentration of 10 µM to the ACSF perfusing the slices. Initial experiments attempted to utilize a within-cell design in which baseline mEPSCs were collected in standard ACSF followed by continued recording in 10 µM cocaine ACSF. However, we found that recordings became extremely unstable during bath application of cocaine, producing low quality data. Thus, we utilized a between-cell experimental design in which slices were bathed in cocaine ACSF for 10 minutes and then switched to perfusing with standard ACSF for recording. Recordings took place up to 1 hour following *ex vivo* drug exposure, and the time elapsed between drug exposure and the beginning of a recording was noted to determine whether the amount of time following drug exposure was a factor in synaptic plasticity.

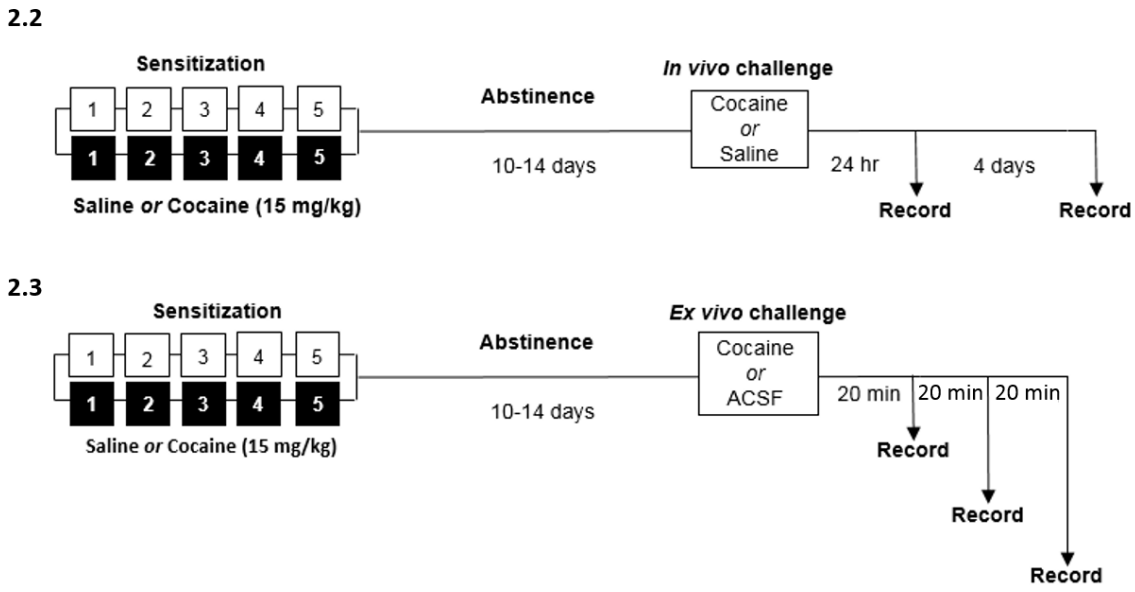

Figure 2.1. Timeline of experimental manipulations. Mice received five once-daily injections of cocaine or saline, followed by 10-14 days of abstinence in their home cages. For *in vivo* challenge experiments, animals received a cocaine challenge injection following the abstinence period. Recording took place 24 hours *or* 5 days following the challenge. For *ex vivo* experiments, brain slices were prepared following the abstinence period. Cocaine challenge was carried out *ex vivo*, after which recordings took place immediately. Recordings were binned into groups according to the time elapsed between *ex vivo* challenge and the start of recording: 20, 40, and 60 minutes.

2.5 Drugs

Lidocaine and picrotoxin were purchased from Sigma Aldrich (St. Louis, MO, USA). Cocaine was obtained from Boynton Health Services Pharmacy (University of Minnesota, Minneapolis, MN, USA). Drugs were prepared in a stock solution at 100 – 1000 times the desired concentration and added into standard ACSF at the final concentration.

2.5 Statistical analysis

All data are presented as group mean ± SEM. Statistical significance was assessed with a Student's t-test, one-way ANOVA, or two-way ANOVA using JMP Pro (SAS, Cary, NC, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Tukey HSD *post hoc* tests were used for pairwise comparisons where appropriate. The threshold for significance was $p<0.05$.

Results

Cocaine-induced potentiation of AMPAR synaptic transmission in the NAc shell is reversed by in vivo cocaine challenge and persists for fewer than 5 days post challenge.

Previous studies examining cocaine-induced AMPAR plasticity in the NAc have shown that the ability of intra-NAc AMPA to induce locomotor activity is decreased 24 hours but not 6 days following a cocaine challenge (Bachtell and Self, 2008), indicating that cocaine challenge-induced plasticity in the NAc is transient in nature. To investigate the duration of AMPAR synaptic plasticity in the NAc shell, we performed *ex vivo* recordings of mEPSCs, a direct measure of synaptic AMPAR function, 24 hours or 5 days following a cocaine challenge injection (given on day 10-14 of abstinence). Twenty-four hours following cocaine challenge, mEPSC amplitude (picoamperes of current, *pA*) and frequency (events per second, *Hz*) were significantly reduced compared to cocaine-treated animals receiving a saline challenge [amplitude (**figure 2.2B**-left): Sal (11.29 ± 0.40, 7 cells/3 animals); Coc (14.50 ± 0.97, 8 cells/5 animals); Coc-coc 24h (10.84 ± 0.42, 9 cells/3 animals); $F_{(3,29)} = 7.72$, p=0.0006); frequency (figure **2.2B**-right): Sal (3.80

± 0.44); Coc (5.52 ± 0.30); Coc-coc 24h (3.55 ± 0.28); $F_{(3,29)} = 6.04$, p=0.0026]. However, 5 days following *in vivo* cocaine challenge, neither mEPSC amplitude (14.60 ± 0.89) nor frequency (5.93 ± 0.80) (9 cells, 3 animals) were significantly different from cocaine-treated controls receiving no challenge injection. Additionally, no differences in AMPAR mEPSC rise time kinetics [Sal (3.26 ± 0.35); Coc (2.70 ± 0.14); Coc-coc 24h (3.33 ± 0.10); Coc-coc 5d (3.00 ± 0.10); $F_{(3,20)}$=0.73, p=0.55, data not shown) or decay time kinetics [Sal (3.24 ± 0.19); Coc (3.73 ± 0.15); Coc-coc 24h (3.93 ± 0.30); Coc-coc 5d (3.93 ± 0.21); $F_{(3,20)}$=2.31, p=0.11, not shown] were observed.

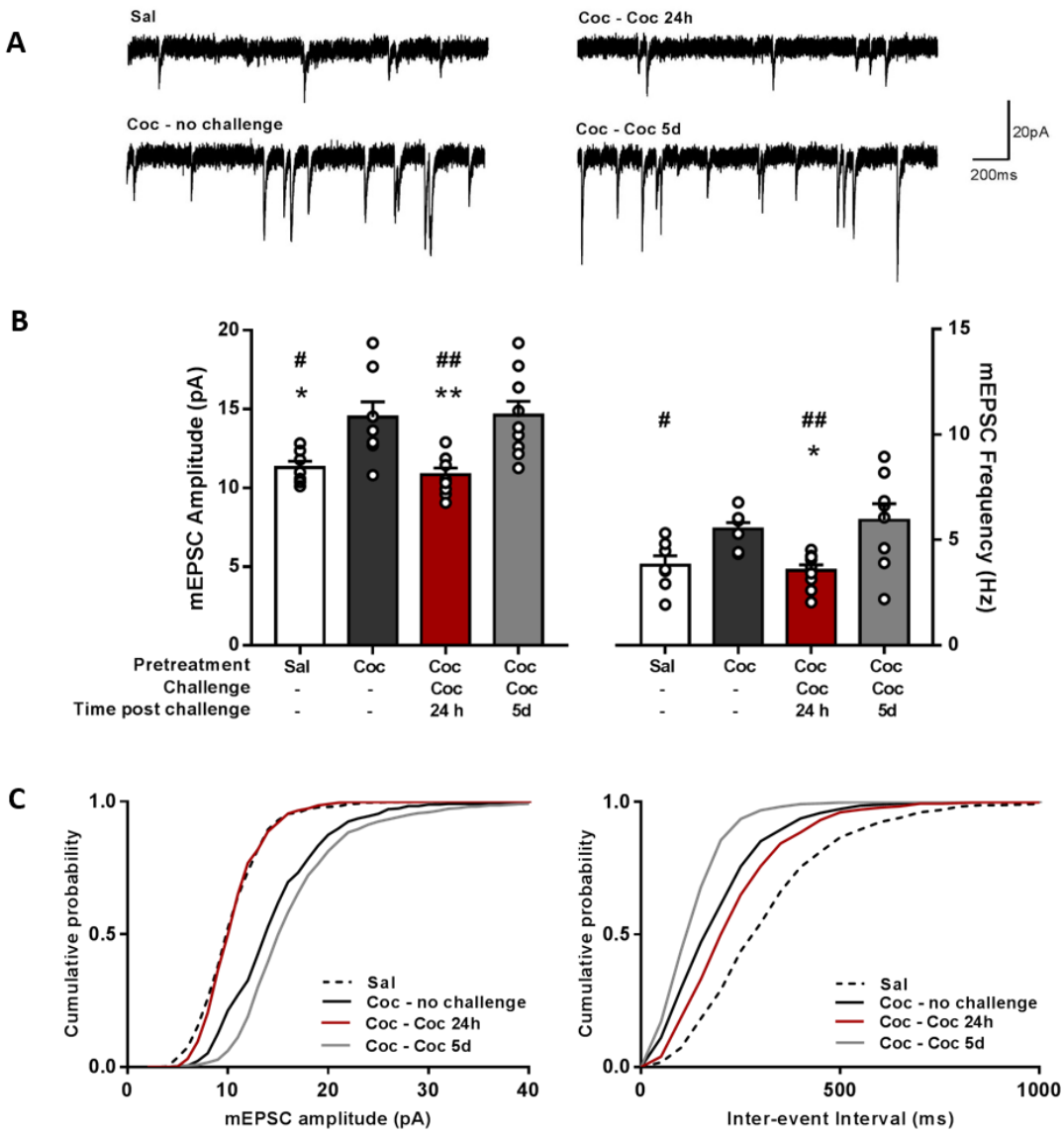

Figure 2.2. Cocaine-induced potentiation of AMPAR synaptic transmission in the NAc shell is reversed by in vivo cocaine challenge and persists for fewer than 5 days post challenge. (A) Representative mEPSC current traces from NAc shell neurons. (B) Mean mEPSC amplitude and frequency in the NAc shell from saline + no challenge (sal), cocaine + no challenge (Coc), cocaine + cocaine challenge at 24h (Coc-coc 24h); cocaine + cocaine challenge at 5d (Coc-coc 5d). (D) Cumulative probability distributions of mEPSC amplitude and inter-event interval from saline + no challenge (Sal), cocaine + no challenge (Coc), cocaine + cocaine challenge at 24h (Coc-coc 24h), cocaine + cocaine challenge at 5d (Coc-coc 5d). All data are presented as mean ± SEM. * p≤0.05 vs. Coc, ** p≤0.01 vs Coc; # p≤0.05 vs Coc-coc 5d, ## p≤0.01 vs Coc-coc 5d.

Ex vivo cocaine re-exposure induces depotentiation of AMPAR signaling in the NAc shell that is maximally expressed within 20 minutes post challenge.

Studies investigating cocaine-induced reversal of synaptic potentiation in the NAc have characteristically observed synaptic plasticity at 2-24 hours following *in vivo* cocaine challenge injection, leaving open the question of how rapidly plasticity is induced by cocaine re-exposure (Bachtell and Self, 2008; Boudreau et al., 2007; Kourrich et al., 2007; Rothwell et al., 2011). To examine this, we utilized a recently developed *ex vivo* model of cocaine re-exposure which our lab has shown to reverse the increase in NAc shell AMPAR transmission produced by repeated *in vivo* cocaine exposure (Jedynak et al., 2016). Following bath application of cocaine, recordings of mEPSCs were performed in the NAc shell for up to 1 hour, with cells binned into 20-, 40-, and 60-min timing groups based on the time elapsed between cocaine challenge and recording. Consistent with previous studies (Jedynak et al., 2016; Kourrich et al., 2007), the amplitude and frequency of mEPSCs were elevated in MSNs from cocaine-treated animals in the NAc shell, indicating potentiation of AMPAR synaptic transmission during abstinence [amplitude (**figure 2.3B**-left): Coc (13.65 ± 0.38, 23 cells/19 animals); frequency (**figure 2.3B**-right): Coc (6.8 ± 0.58)]. Following *ex vivo* challenge at all time intervals, we observed a significant decrease in mEPSC amplitude and frequency, with a ~30% reduction in AMPAR-mediated signaling observed equally at all three post-challenge timepoints [amplitude (**figure 2.3B**-left): 20 min (9.62 ± 0.40, 7 cells/6 animals); 40 min (10.30 ± 0.65, 6 cells/6 animals); 60 min (10.46 ± 0.60, 7 cells/6 animals); $F_{(3,46)}$ =14.36, p<0.0001; frequency (**figure 2.3B**-right): 20 min (3.95 ± 0.49);

40 min (3.44 ± 0.22); 60 min (3.44 ± 0.37); $F_{(3,46)}$=9.03, p<0.0001]. No differences in rise time kinetics (milliseconds, ms) [Coc (3.29 ± 0.08); 20 min (3.53 ± 0.19); 40 min (3.60 ± 0.20); 60 min (3.58 ± 0.23); $F_{(3,32)}$=1.21, p=0.32, data not shown) or decay time kinetics (milliseconds, ms) [Coc (4.11 ± 0.15); 20 min (4.12 ± 0.21); 40 min (3.72 ± 0.38); 60 min (4.24 ± 0.36); $F_{(3,34)}$=0.62, p=0.61, not shown] were observed in AMPAR mEPSCs.

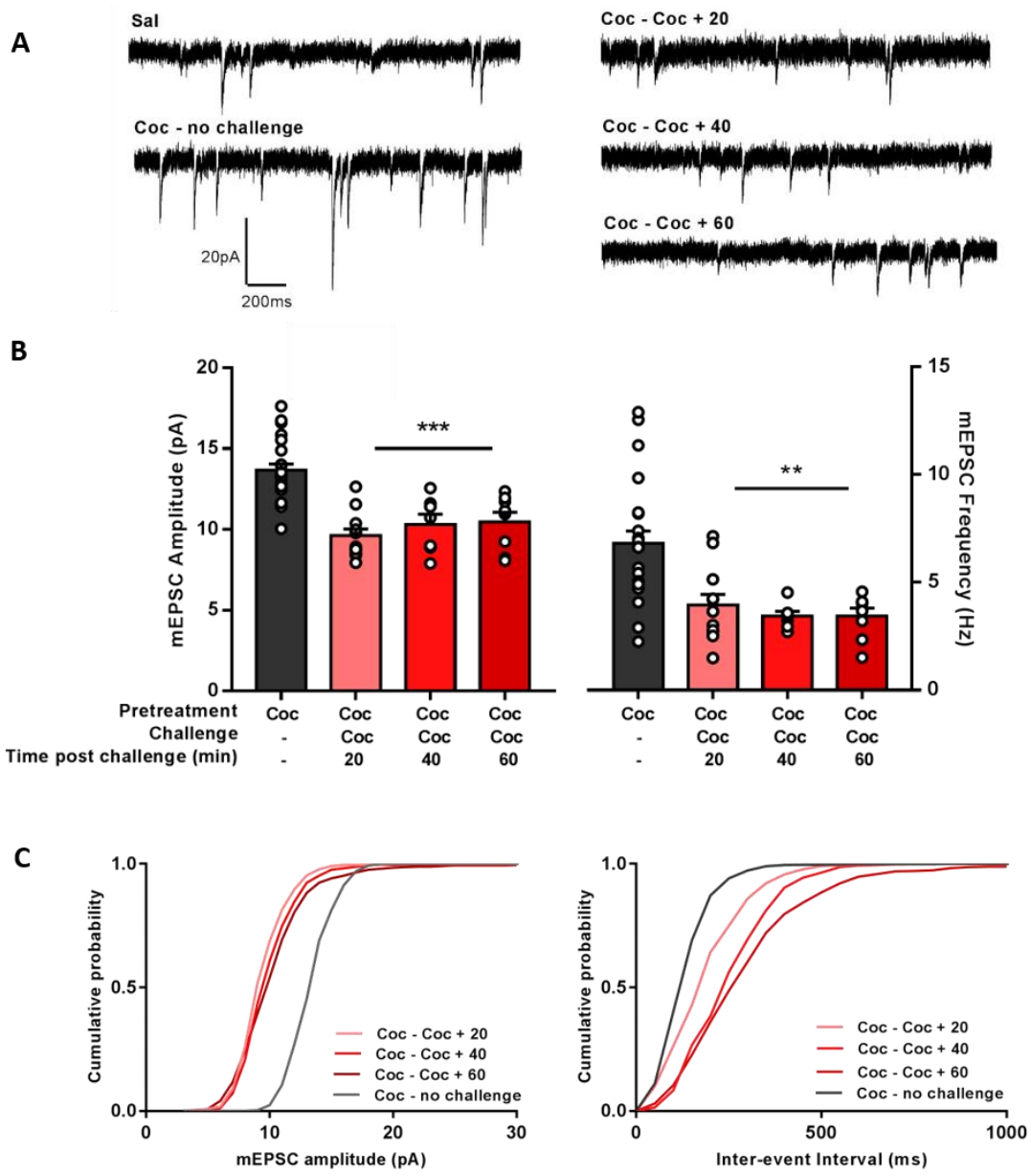

Figure 2.3 *Ex vivo* cocaine re-exposure induces depotentiation of AMPAR signaling in the NAc shell that is maximally expressed within 20 minutes post challenge. (A) Representative miniature excitatory postsynaptic current (mEPSC) traces from NAc shell neurons. (B) Mean mEPSC amplitude and frequency in the NAc shell from cocaine + no challenge (Coc), cocaine + cocaine 20 min post challenge (Coc-coc 20), cocaine + cocaine 40 min post challenge (Coc-coc 40), cocaine + cocaine 60 min post challenge (Coc-coc 60). (D) Cumulative probability distributions of mEPSC amplitude and inter-event interval from cocaine + no challenge (Coc-sal), cocaine + cocaine 20 min post challenge (Coc-coc + 20), cocaine + cocaine 40 min post challenge (Coc-coc + 40), cocaine + cocaine 60 min post challenge (Coc-coc + 60). All data are presented as mean ± SEM. ** $p \leq 0.01$ vs. Coc, *** $p \leq 0.001$ vs Coc.

Discussion

The current study investigated the temporal profile of bidirectional AMPAR plasticity in the NAc shell following re-exposure to cocaine during abstinence. Here, we find that *ex vivo* cocaine re-exposure rapidly induces depotentiation of AMPAR-mediated signaling in the NAc shell on a timescale of less than 20 minutes. Furthermore, this plasticity persists for at least 24 hours but not five days following a cocaine challenge injection, after which synapses return to "repotentiated" levels of synaptic strength. These findings have implications for understanding how the timing of drug use and subsequent re-use following periods of abstinence can differentially alter excitatory synaptic transmission in the NAc.

Our finding that NAc shell synapses are repotentiated within five days following renewed cocaine exposure is consistent with previous work demonstrating a re-emergence of potentiated AMPAR signaling following a similar time interval after cocaine re-exposure: the ability of an intra-NAc infusion of AMPA to augment locomotor activity is abolished 24 hours following cocaine challenge, but regains efficacy six days after the challenge injection (Bachtell and Self, 2008). This "rebound effect" was similarly observed in a study reporting that surface expression of AMPARs in the NAc is decreased 24 hours after cocaine challenge but returns to pre-challenge levels of expression after seven days of abstinence (Ferrario et al., 2009). Coupled with our data, these observations might suggest that the potentiation of AMPAR synaptic strength evoked by the initial repeated cocaine treatment regimen produces plasticity that appears to be more durable and longer-lasting than depotentiation induced by cocaine re-

exposure, such that cocaine re-exposure temporarily induces a relative reduction in synaptic strength, after which synapses return to potentiated levels.

Rapid shifts in synaptic AMPAR content in the NAc could reflect the engagement of homeostatic mechanisms functioning to counteract the alterations in glutamatergic signaling that arise during periods of abstinence and drug re-exposure. Indeed, increased surface expression of post-synaptic AMPARs has been suggested to be a mechanism expressed to maintain synaptic glutamate homeostasis, counteracting the decreased glutamate tone and reduced neuronal excitability in the NAc that emerges during abstinence from chronic cocaine treatment cocaine (Baker et al., 2003; Kourrich and Thomas, 2009; Kourrich et al., 2007). Transient elevations in glutamate during cocaine re-exposure (Baker et al., 2003) would be expected to disrupt this balance, eliciting downregulation of AMPAR signaling. Our data indicates that excitatory synapses in the NAc shell appear to recover and regain their heightened AMPAR sensitivity within a relatively short period following cocaine re-exposure.

Here, we found that cocaine re-exposure engages mechanisms of plasticity that occur rapidly and are maximally expressed in within 20 minutes. While the rapid induction of cocaine-evoked plasticity is striking, it is consistent with previous literature demonstrating that *ex vivo* cocaine is capable of dynamically modifying AMPAR synaptic strength in the NAc on a short timescale of 10-20 minutes (Dobbs et al., 2016; Harvey and Lacey, 1996; Nicola, 1996). In terms of this study, the induction of plasticity on a brief timescale constrains the potential molecular and cellular mechanisms that might be engaged by cocaine re-exposure. Regulation of AMPAR endocytosis machinery

likely mediates the rapid alterations in AMPAR synaptic levels (Bachtell and Self, 2008; Brebner et al., 2005). While changes in gene expression are unlikely to occur on such a short timescale, on-demand protein translation occurring locally in dendrites has been shown to mediate rapid increases in AMPAR endocytosis culminating in a reduction in synaptic strength in the NAc (Lüscher and Huber, 2010; Waung et al., 2008), consistent with previous findings from our lab demonstrating that rapid protein synthesis is necessary for cocaine-induced synaptic depotentiation (Chapter 1). Thus, the rapid induction of plasticity that we observe here likely engages common mechanisms for dynamically regulating excitatory synaptic strength in the NAc.

In conclusion, these findings acknowledge the existence of multiple overlapping forms of plasticity at NAc synapses that likely serve to regulate and update synaptic strength as a consequence of varied drug experiences. Initially, abstinence from repeated cocaine initiates mechanisms that promote enhanced glutamatergic synaptic transmission, which develops gradually over time and is observed only during late abstinence (Kourrich et al., 2007; Ortinski et al., 2012; Wolf, 2010). The mechanisms and the time course of this plasticity are distinct from the rapid reduction in AMPAR-mediated synaptic transmission induced by cocaine re-exposure, demonstrating that synapses altered by cocaine exposure remain plastic and are capable of undergoing further modifications by drug re-exposures. Synaptic depotentiation is constrained to a relatively short amount of time, after which NAc shell synapses return to a potentiated state. This modification of synapses through potentiation, depotentiation, and re-potentiation might

represent how neural circuits continually update and maintain relevant drug-related memory traces at activated synapses.

Chapter 3: Endogenous dopamine and endocannabinoid signaling mediate reversal of AMPAR synaptic potentiation in the NAc shell.

Introduction

Neuromodulators are key regulators of neuronal activity, modifying the excitability of cells and shaping synaptic input to alter large-scale network dynamics and subsequently influence behavioral and cognitive states (Lee and Dan, 2012; Melis et al., 2014). Furthermore, neuromodulators regulate critical aspects of synaptic plasticity through a number of mechanisms including adjusting the threshold for plasticity induction, regulating the maintenance of long-term forms of plasticity, and modifying a host of cellular mechanisms mediating synaptic plasticity (Mameli et al., 2009; Marsicano and Lutz, 2006; Melis et al., 2014). Given the critical functions of neuromodulators on neural activity and plasticity throughout the brain, dysfunctional neuromodulation may play a key role in a number of disease conditions, including addiction (Fernández-Ruiz et al., 2010; Lüscher and Huber, 2010).

Dopamine modulation of glutamatergic synaptic plasticity

Prominent among these neuromodulators, dopamine (DA) regulates glutamatergic synaptic transmission in the striatum, gating the excitability, activity, and induction of plasticity at NAc MSN synapses (Calabresi et al., 2007; Gerfen and Surmeier, 2011; Tritsch and Sabatini, 2012). DA receptors are located on both pre-synaptic afferents to the NAc and on MSNs themselves, and thus are in a prime position to regulate mechanisms of plasticity at NAc glutamatergic synapses. Dissecting the mechanisms by

which DA modulates glutamatergic synaptic strength is complex since dopamine can exert divergent effects on cells depending on the DA receptor type activated: D1-class (D1, D5: G_s-coupled GPCR) or D2-class (D2, D3, D4: $G_{i/o}$-coupled). One mechanism by which DA could modulate glutamatergic synaptic transmission is by altering the number, function, or trafficking of AMPARs in the synapse. Several studies utilizing cultured MSNs have reported that acute activation of DA D1-class receptors increases cell surface expression of GluA1-containing AMPARs through PKA-dependent phosphorylation (Cepeda et al., 1993; Chao et al., 2002; Mangiavacchi and Wolf, 2004) and potentiates AMPAR-mediated currents (André et al., 2010; Price et al., 1999), indicating that D1 activation can promote the trafficking and insertion of AMPARs at the synapse. Conversely, D2 stimulation decreases AMPAR expression levels and reduces AMPAR transmission (André et al., 2010; Hernández-Echeagaray et al., 2004; Sun et al., 2005).

Dopamine D1 and D2 receptor mechanisms therefore appear to oppositely regulate AMPAR synaptic strength. However, repeated exposure to exogenous DA, modeling the increased synaptic availability of DA induced by cocaine, abolishes the ability of D1 stimulation to promote increased AMPAR function (Sun et al., 2008). Similarly, PKA-mediated phosphorylation of AMPAR subunits, a necessary step in membrane trafficking, is strongly increased in the NAc following acute cocaine exposure but only weakly activated following several weeks of cocaine self-administration (Bibb et al., 2001; Edwards et al., 2007), indicating that sustained elevations in DA alter the normal function of DA receptors to modulate glutamatergic synaptic strength in a time-sensitive manner. Given the critical role that DA plays in reward-related learning,

motivation, and addiction (Kauer and Malenka, 2007; Wheeler and Carelli, 2009), these studies highlight a potential role for DA receptors in mediating AMPAR plasticity in the NAc modified by cocaine experience.

Modulatory functions of the endocannabinoid system

The endocannabinoid (eCB) system is another critical neuromodulator of synaptic activity and plasticity throughout the brain. eCBs are lipophilic membrane-derived molecules, of which anandamide and 2-arachidonoyl glycerol (2-AG) are the most abundant and best-characterized eCBs in the CNS. eCBs are synthesized on-demand from membrane-derived lipid precursors in response to post-synaptic activation of G_q-coupled Group I metabotropic glutamate receptors (mGluRs) or elevations in post-synaptic calcium (Castillo et al. 2012). Landmark studies characterizing the eCB system revealed that once released from the post-synaptic cell, eCBs diffuse via retrograde action to cannabinoid type 1 (CB1) receptors, $G_{i/o}$-coupled GPCRs located on pre-synaptic axon terminals that suppress neurotransmitter release when activated (Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001). CB1 receptors are highly expressed at excitatory and inhibitory synapses throughout the CNS but are particularly abundant in regions of the mesocorticolimbic dopamine system associated with reward, motivation, and cognition, including the striatum (Herkenham et al., 1991; Mátyás et al., 2006). Within the striatum, eCB signaling has emerged as a dominant mechanism for reducing synaptic strength at excitatory synapses through several forms of short-term and long-term synaptic depression (Lovinger and Mathur, 2017; Robbe et al., 2003; Zlebnik and Cheer, 2016).

The retrograde signaling mechanism of the eCB system provides a "gain control" negative feedback loop through which post-synaptic neuronal activity can modify the level of synaptic input at afferents onto MSNs, finely adjusting synaptic strength.

Accumulating evidence indicates that eCB signaling at the CB1 receptor plays a central role in modulating reward, motivation, and addiction (Olière et al., 2013; Parsons and Hurd, 2015). NAc shell CB1 signaling is necessary for the expression of cocaine-primed behavioral sensitization, without appearing to play a role in the induction phase of sensitization (Filip et al., 2006; Gerdeman et al., 2007; Ramiro-Fuentes and Fernandez-Espejo, 2011). In studies utilizing the drug self-administration model, CB1 signaling is necessary for cocaine- (Xi et al., 2006), cue- (De Vries et al., 2001), and stress-potentiated (McReynolds et al., 2016) reinstatement of cocaine seeking following a period of abstinence or extinction, without impacting acquisition of drug seeking. Additionally, antagonism of NAc CB1 receptors reduces breakpoints under a progressive ratio schedule of cocaine seeking (Orio et al., 2009), suggesting that the eCB system might mediate increased motivation to seek drug over an extended period of time. These studies broadly indicate a pattern whereby CB1 signaling is a critical factor in the persistence of cocaine seeking over a protracted period of drug administration and subsequent abstinence or extinction, while being minimally engaged by more short-term drug exposure paradigms. The ability of the eCB system to selectively alter persistent drug-seeking behavior furthermore suggests the modulation of neuroadaptations set in place by chronic drug experience, making this system a prime candidate in mediating cocaine-evoked alterations in glutamatergic synaptic strength.

In this study, the role of dopamine and eCB signaling in cocaine-induced depotentiation of glutamatergic synaptic strength in the NAc was investigated, examining changes in AMPAR-mediated signaling induced by cocaine experience. As in previous studies, we employed an *ex vivo* cocaine challenge model to allow for pharmacological isolation of the cellular signaling mechanisms of NAc synaptic plasticity.

Materials and methods

Animals

Adult male (P49-70) C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine, USA) were used. All animals were group-housed in a temperature- and humidity-controlled environment on a 12 hr light/12 hr dark cycle, with food and water available *ad libitum.* All experiments were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Behavioral sensitization

Prior to testing, mice were habituated to experimenter handling, *i.p*. injections, and the testing environment (individual activity boxes, 8.5 x 17.5 x 9 in) over two days. On five consecutive testing days, mice were habituated to the testing environment for 30 minutes (min) before receiving an intraperitoneal injection of either cocaine (15 mg/kg) or saline. Mice were immediately placed back into the testing environment, and their activity was monitored for 90 min using a video-based tracking system (Any-Maze, Stoelting, WI, USA). Animals were returned to their home cages at the end of each

testing period. Following the last day of testing, animals remained in their home cages in the colony for 10 – 14 days before electrophysiology experiments were performed. To determine whether repeated cocaine treatment produced behavioral sensitization, the locomotor activity (distance traveled) during the first 30 minutes of the session on Day 1 and Day 5 was compared, pooling all cocaine-treated animals. A t-test was used to confirm a significant difference in locomotor activity between Day 1 and Day 5, indicating the development of behavioral sensitization to cocaine. Animals that did not fit these criteria were excluded from recording experiments.

Slice electrophysiology

Following 10–14 days of abstinence from cocaine treatment, mice were anesthetized with isoflurane, decapitated, and the brain rapidly removed. Parasagittal slices (240 μm) containing the nucleus accumbens shell were prepared in 2-4°C sucrose-containing artificial cerebrospinal fluid (ACSF), after which slices rested for at least 30 min in standard ACSF at room temperature. To assess AMPAR-mediated excitatory synaptic transmission, cells were voltage-clamped at -80 mV using an Axon Instruments MultiClamp 700A (Molecular Devices, Sunnyvale, CA, USA). Data were filtered at 2 kHz, digitized at 5 kHz, and collected using custom Igor Pro software (Wavemetrics, Lake Oswego, OR, USA). MiniAnalysis software (Synaptosoft, Decatur, GA, USA) was used offline to analyze mEPSC amplitude, frequency, rise time, and decay time. To determine the rise time and decay time, two exponentials were fit to the rise time or decay time of mEPSCs using the MiniAnalysis curve-fitting function, and the time

constant was calculated between 10-90% of peak. For a more detailed description of slice preparation and recording methods, see Chapter 1.

Ex vivo drug application

Drugs were prepared in a stock solution and added at the final concentration to the ACSF perfusing the slices. A between-cell experimental design was utilized in which slices were bathed in the drug or control ACSF prior to recording synaptic activity. During these experiments, slices were exposed to one of the following conditions:

1. No challenge (ACSF only)
2. *Ex vivo* cocaine challenge (10 µM; 10 min)
3. *Ex vivo* flupenthixol (non-specific dopamine receptor antagonist; 20 µM; 10 min) *or* SR141716A (CB1 antagonist/inverse agonist; 1 µM; 10 min) alone;
4. *Ex vivo* flupenthixol *or* SR141716A alone (10 min) followed by flupenthixol *or* SR141716A in the presence of 10 µM cocaine (10 min);
5. *Ex vivo* apomorphine (non-specific dopamine receptor agonist; 1 µM; 10 min) *or* WIN 55,212-2 (CB1 receptor agonist; 1 µM; 20 min) alone.

Following the timed *ex vivo* drug exposure(s), the perfusion was switched back to standard ACSF for recording. Recordings took place up to 1 hour following *ex vivo* drug exposure.

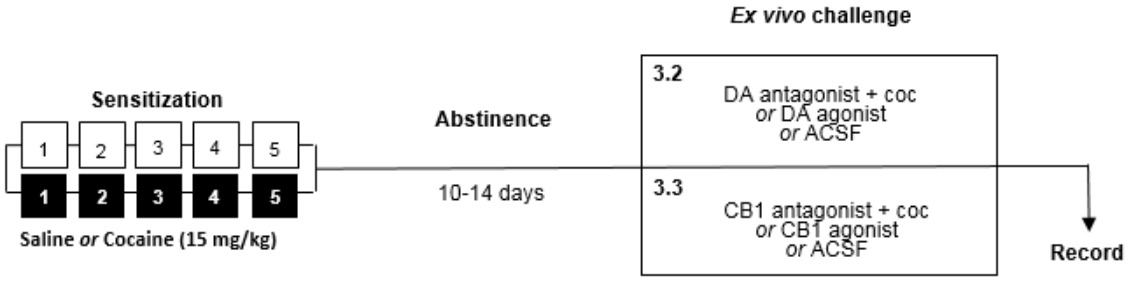

Figure 3.1. Timeline of experimental manipulations. Mice received five once-daily injections of cocaine or saline, followed by 10-14 days of abstinence in their home cages. Brain slices were prepared following the abstinence period, and cocaine challenge was performed *ex vivo*: slices were exposed to 10 µM cocaine in the perfusing ACSF for 10 minutes, followed by electrophysiological recording in standard ACSF. To examine DA receptor signaling, the DA antagonist flupenthixol (10 µM) was added to the bath before cocaine challenge, or a DA receptor agonist apomorphine (1 µM) was substituted in place of the cocaine challenge. To examine CB1 signaling, the antagonist SR141716A (1 µM) was added to the bath before cocaine challenge, or the agonist WIN 55,212-2 was used in place of cocaine. Recording took place immediately following drug challenges.

Drugs

Apomorphine, flupenthixol, SR141716A, and WIN 55,212-2 were purchased from Tocris Bioscience (Bristol, United Kingdom). Lidocaine and picrotoxin were purchased from Sigma Aldrich (St. Louis, MO, USA). Cocaine was obtained from Boynton Health Services Pharmacy (University of Minnesota, Minneapolis, MN, USA). Drugs were prepared in a stock solution at 100 – 1000 times the desired concentration and added into standard ACSF at the final concentration. SR141716A stock was dissolved in DMSO, and WIN 55,212-2 stock was dissolved in ethanol. The final concentrations of DMSO or ethanol were <0.01%.

Statistical analysis

All data are presented as group mean ± SEM. Statistical significance was assessed with a Student's t-test, one-way ANOVA, or two-way ANOVA using JMP Pro (SAS, Cary, NC, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Tukey HSD *post hoc* tests were used for pairwise comparisons where appropriate. The threshold for significance was $p<0.05$.

Results

Dopamine receptor activation is necessary for cocaine-induced synaptic depotentiation.

In the striatum, dopamine plays a critical role in regulating the induction of synaptic plasticity by modulating excitatory synaptic transmission through several mechanisms, including modification of post-synaptic neurotransmitter receptors (O'Donnell, 2003; Surmeier et al., 2007; Tritsch and Sabatini, 2012). Several studies indicate that dopamine receptor activation promotes increased surface expression of MSN AMPARs (Snyder et al., 2000; Wolf, 2010). Thus, we hypothesized that dopamine receptor activation provoked by cocaine re-exposure might participate in the reversal of enhanced AMPAR synaptic transmission in the NAc shell.

To examine this, we applied the non-specific dopamine antagonist flupenthixol *ex vivo* prior to cocaine challenge to determine whether dopamine receptor activation is necessary for the induction of AMPAR synaptic depotentiation. A non-specific dopamine receptor antagonist was chosen for these studies, as our goal was to assess AMPAR function in NAc MSN subpopulations as a whole, rather than differentiate between effects that may selectively occur on MSNs expressing select DA receptor sub-types. As

previously demonstrated, abstinence (10-14 d) from repeated cocaine potentiated mEPSC amplitude (picoamperes of current, *pA*), which was reversed by *ex vivo* cocaine bath challenge [figure **3.2B**-left: Sal (10.19 ± 0.53, 8 cells/6 animals); Coc (13.09 ± 0.46, 11 cells/10 animals); Coc-coc (10.04 ± 0.40, 9 cells/6 animals); $F_{(4,37)} = 10.39$, $p<0.0001$]. Similarly, mEPSC frequency (events per second, *Hz*) was increased during withdrawal from cocaine but reversed by *ex vivo* cocaine challenge [figure **3.2B**-right: Sal (4.08 ± 0.53); Coc (6.87 ± 0.84); Coc-coc (4.23 ± 0.42); $F_{(4,37)} = 4.59$, $p=0.0041$]. However, bath application of flupenthixol (20 µM) prior to *ex vivo* cocaine challenge blocked the cocaine-induced reduction in mEPSC amplitude (12.42 ± 0.70) and frequency (6.410 ± 0.86) (7 cells/4 animals). Exposure to flupenthixol alone had no baseline effects on mEPSC amplitude or frequency in saline-treated (amplitude: 10.53 ± 0.89; frequency: 4.81 ± 0.76, 7 cells/6 animals, data not shown) or cocaine-treated animals (amplitude: 12.08 ± 0.64; frequency: 5.36 ± 0.67, 6 cells/5 animals, not shown). In addition, there were no differences in the rise time kinetics [Sal (3.28 ± 0.14); Coc (3.18 ± 0.08); Coc-coc (3.61 ± 0.19); Coc-Flp-coc (3.31 ± 0.13); $F_{(4,34)}=1.70$, $p=0.17$, not shown] or decay time kinetics [Sal (3.62 ± 0.22); Coc (3.89 ± 0.16); Coc-coc (3.79 ± 0.27); Coc-Flp-coc (4.17 ± 0.23); $F_{(4,34)}=0.80$, $p=0.53$, not shown] of AMPAR mEPSCs evident among the groups.

Dopamine receptor activation is sufficient to drive depotentiation of AMPAR synaptic transmission in the NAc shell.

Given that activation of DA receptors has been shown to mediate the induction of striatal LTD (Calabresi et al., 2007; Kreitzer and Malenka, 2005), we next investigated whether dopamine receptor activation is sufficient to drive reductions in AMPAR signaling produced by cocaine and subsequent abstinence. *Ex vivo* application of the non-specific dopamine receptor agonist apomorphine (1 μM) reduced mEPSC amplitude (figure **3.2B**-left; 9.33 ± 0.40) and frequency (figure **3.2B**-right; 3.61 ± 0.36) (6 cells/4 animals) compared to cocaine + no challenge controls. Apomorphine application in saline animals had no effect on baseline mEPSC amplitude (9.62 ± 0.63) or frequency (3.37 ± 0.61) (6 cells/4 animals). AMPAR mEPSC rise time (3.56 ± 0.21) and decay time kinetics (3.91 ± 0.26) were unchanged by apomorphine. Taken together, these data demonstrate that dopamine receptor activation is both necessary and sufficient for AMPAR synaptic depotentiation elicited by re-exposure to cocaine, demonstrating a central role for dopamine receptors in mediating this form of plasticity.

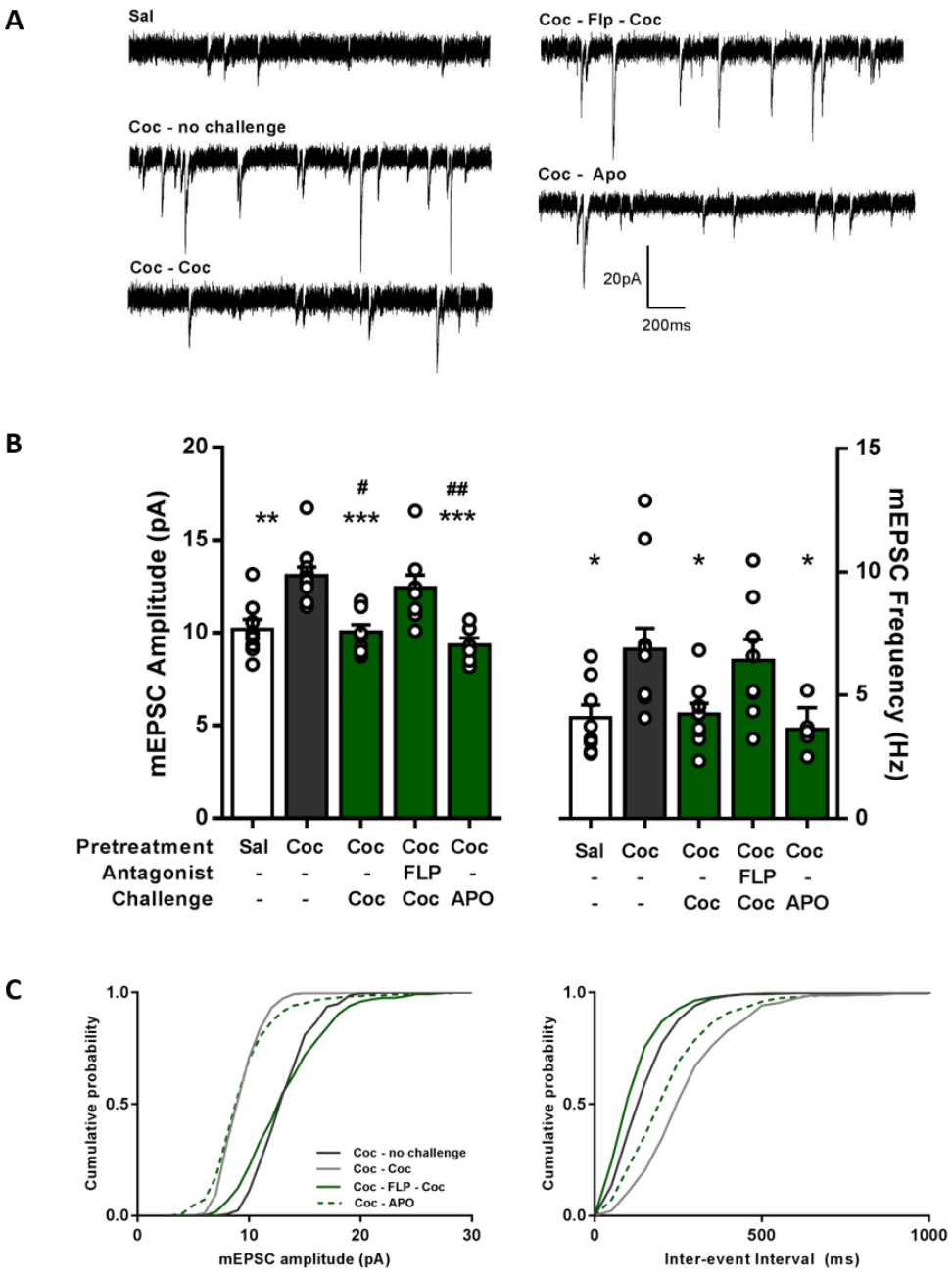

Figure 3.2. Dopamine receptor activation is necessary for cocaine-induced depotentiation of NAc shell AMPAR signaling. (A) Representative mEPSC current traces from NAc shell neurons. (B) Mean mEPSC amplitude and frequency in the NAc shell from saline (Sal), cocaine + no challenge (Coc), cocaine + cocaine challenge (Coc-coc), cocaine + flupenthixol/cocaine (Coc-Flp-Coc), cocaine + apomorphine (Coc-Apo). (C) Cumulative probability distributions of mEPSC amplitude and inter-event interval of treatment groups. All data are presented as mean ± SEM. *p<0.05 vs. Coc, ** p<0.01 vs. Coc, *** p<0.001 vs Coc; # p<0.05 vs. Coc-Flp-coc, ## p<0.01 vs. Coc-Flp-coc.

CB1 receptor activation is necessary for cocaine-induced synaptic depotentiation.

Within the NAc, endogenous cannabinoids play a central role in modulating glutamatergic synaptic transmission (Lüscher and Huber, 2010; Robbe et al., 2002). Located predominantly on axon terminals contacting MSNs, activation of CB1 receptors promotes suppression of neurotransmitter release following activation of post-synaptic signaling mechanisms (Robbe et al., 2001; Hoffman and Lupica, 2001; Robbe et al., 2003). To test whether CB1 receptor activation mediates cocaine-induced reductions in AMPAR synaptic transmission, we bath applied the CB1 antagonist/inverse agonist SR141716A during an *ex vivo* cocaine challenge. While bath application of SR141716A (1 μM) in the presence of *ex vivo* cocaine produced only a modest blockade of the cocaine-induced reductions in mEPSC amplitude (figure **3.3B**-right; Sal (10.32 ± 0.35, 8 cells/7 animals); Coc (14.17 ± 0.59, 12 cells/9 animals); Coc-coc (9.76 ± 0.52, 10 cells/6 animals); Coc-SR-Coc (11.89 ± 2.47, 15 cells/10 animals); $F_{(4,48)}$=11.31, p<0.0001), it robustly blocked the decrease in mEPSC frequency (figure **3.3B**-right; Sal (3.50 ± 0.38); Coc (6.73 ± 0.82); Coc-coc (3.26 ± 0.28); Coc-SR-coc (6.36 ± 0.62); $F_{(4,48)}$ = 6.73, p=0.0002). No changes in AMPAR rise time kinetics [Sal (3.75 ± 0.17); Coc (3.30 ± 0.09); Coc-coc (3.53 ± 0.17); Coc-SR-coc (3.18 ± 0.16), not shown] or decay time kinetics [Sal (5.10 ± 0.32); Coc (4.36 ± 0.23); Coc-coc (4.33 ± 0.27); Coc-SR-coc (4.01 ± 0.26), not shown] were observed. Bath application of SR141716A produced a slight elevation of baseline mEPSC frequency in both saline and cocaine pre-treated animals, but this effect was not significant [Sal-SR (5.09 ± 1.12, 6 cells/4 animals); Coc-SR (7.91 ± 1.36, 6 cells/5 animals), data not shown].

Activation of NAc CB1 receptors promotes AMPAR synaptic depotentiation.

We next asked whether activation of CB1 receptors is sufficient to induce synaptic depotentiation in the NAc shell. While several studies have reported that activation of CB1 receptors in the striatum is sufficient to induce eCB-mediated LTD (Kreitzer and Malenka, 2005; Robbe et al., 2001), it is not clear whether these mechanisms remain intact or are altered following cocaine exposure. To investigate this, we substituted the *ex vivo* cocaine challenge with bath application of the CB1 receptor agonist WIN 55,212-2 *ex vivo.* Application of WIN 55,212-2 (1 μM) was sufficient to induce depotentiation of mEPSC amplitude (figure **3.3B**-left; 9.69 ± 0.25, 8 cells/4 animals) and frequency (figure **3.3B**-right; 3.99 ± 0.73) in slices from animals abstinent from cocaine, without altering basal mEPSC amplitude (10.38 ± 0.72) or frequency (3.38 ± 0.51) in saline-treated animals (7 cells/3 animals). No changes in mEPSC rise time (3.72 ± 0.23) or decay time (4.76 ± 0.27) kinetics were detected. Our results demonstrate that activating CB1 receptors during abstinence from repeated cocaine is sufficient to depotentiate AMPAR-mediated synaptic transmission in the NAc.

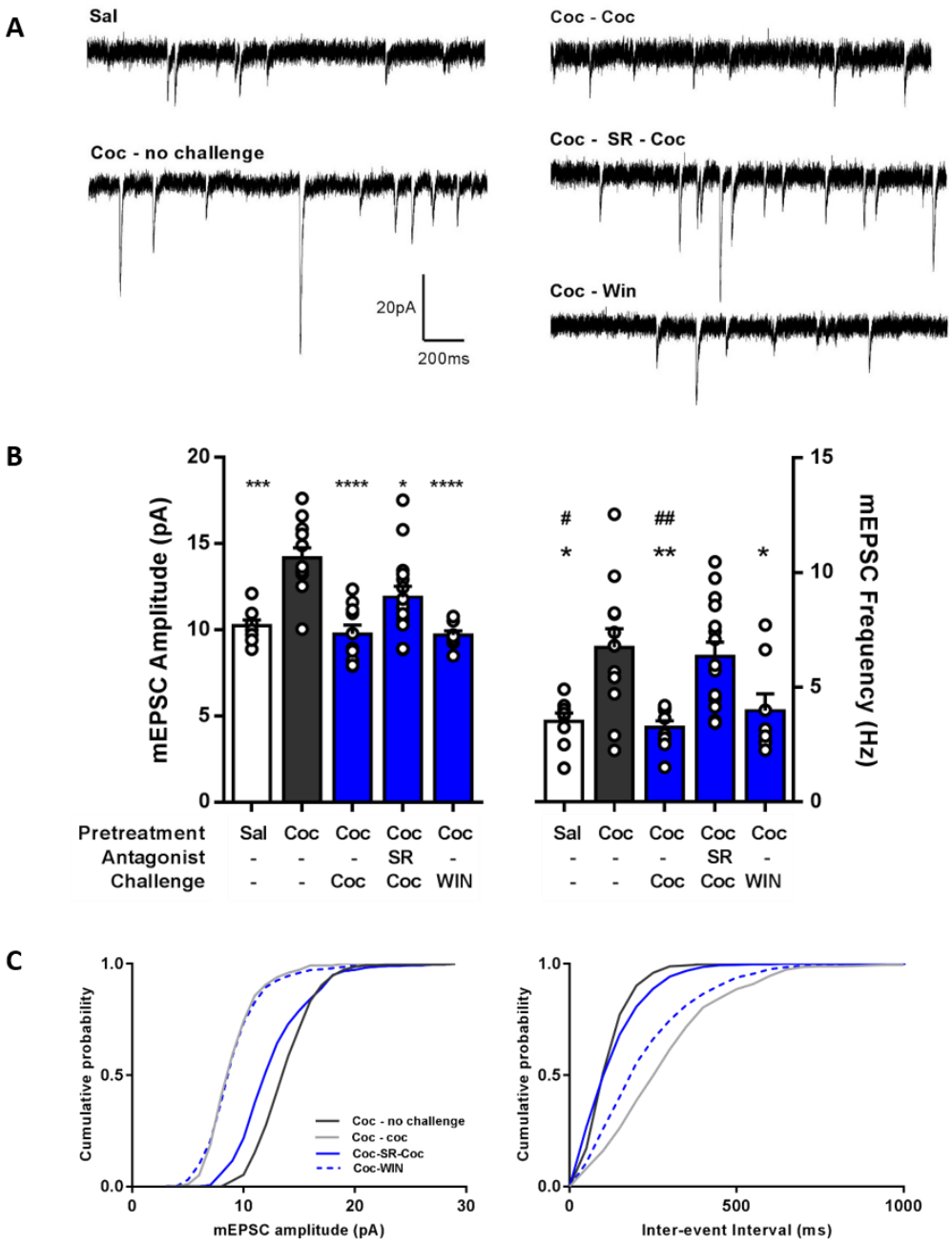

Figure 3.3 CB1 receptor activation is necessary for cocaine-induced synaptic depotentiation. (A) Representative mEPSC current traces from NAc shell neurons. (B) Mean mEPSC amplitude and frequency in the NAc shell from saline (Sal), cocaine + no challenge (Coc), cocaine + cocaine challenge (Coc-coc), cocaine + SR141716A/cocaine (Coc-SR-Coc), cocaine + Win 55,212-2 (Coc-Win). (C) Cumulative probability distributions of mEPSC amplitude and inter-event interval of treatment groups. All data are presented as mean ± SEM. *p<0.05 vs. Coc, ** p<0.01 vs. Coc, *** p<0.001 vs Coc, **** p<0.0001 vs. Coc; # p<0.05 vs. Coc-SR-coc, ## p<0.01 vs. Coc-SR-coc.

Discussion

Dopamine modulation of excitatory synaptic transmission

Dopamine has been well-demonstrated to play an important role in motivated behavior and plasticity within the striatum. Exposure to cocaine *in vivo* (Di Chiara and Imperato, 1988) and *ex vivo* (Hoffmann et al., 2012; Kelly and Wightman, 1987) increases the synaptic availability of dopamine within the NAc. Dopamine action at receptors located both on pre-synaptic axon terminals and on MSNs in the NAc modulates the activity of cells and furthermore gates the induction of plasticity at these synapses by influencing synaptic integration, neurotransmitter release properties, trafficking of post-synaptic receptors, and membrane excitability through complex intercellular mechanisms (Tritsch and Sabatini, 2012). Activation of DA receptors thus acts to "tune" cells to glutamatergic input, thereby altering the dynamics of circuits mediating reward-related behavior. However, DA modulation of excitatory synaptic strength is greatly altered by cocaine experience. While increased dopamine release elicited by natural rewards is tightly controlled by synaptic re-uptake mechanisms, cocaine and other psychostimulants elicit large increases in extracellular dopamine that are unregulated due to inhibition of re-uptake transport. Thus, cocaine exposure produces persistent activation of DA receptors, which is likely to have sustained, inappropriate effects on modulation of glutamatergic transmission, such as altered AMPAR trafficking or desensitization of receptors (Wolf, 2010). The outcome of such intense dopamine stimulation produces strong associative learning, motivating drug-seeking behavior.

Cocaine-induced alterations in dopamine receptor signaling mechanisms

Evidence suggests that long-term exposure to cocaine also alters dopamine receptor signaling mechanisms. One way that cocaine disrupts normal dopamine signaling mechanisms is through disrupting the balance between D1 and D2 receptor activity (Walters et al, 1987). These two receptor sub-types mediate opposing effects on adenylyl cyclase stimulation, activation of PKA, and subsequent triggering of downstream cellular effectors, with D1 receptors generally activating this pathway and D2 receptors suppressing it. Following chronic cocaine exposure, the equilibrium appears to shift toward D1 activation: D1 receptors (Anderson and Pierce, 2005) and cAMP production (Terwilliger et al., 1991) are upregulated, while expression of $G_{i/o}$-coupled proteins is significantly reduced (Nestler et al., 1990; Terwilliger et al., 1991). At the same time, a number of studies have observed that while D1 receptor signaling is able to modulate PKA-dependent trafficking of AMPARs acutely, repeated dopamine or cocaine exposure abolishes this function (Sun et al., 2008), likely due to a reduction in overall PKA activity (Bibb et al., 2001; Edwards et al., 2007). Prolonged D1 stimulation may therefore produce "tolerance" of these signaling mechanisms at the intercellular level, possibly due to desensitization or adaptations of D1 receptor signaling mechanisms. Thus, up-regulated D1 receptors might reflect compensatory mechanisms balancing out the effects of chronic cocaine exposure.

An alternative view is that upregulated D1 receptor signaling mechanisms prime synapses to respond to fluctuations in glutamate signaling (Wolf, 2010). Indeed, D1 receptor stimulation has been well-demonstrated to promote AMPAR trafficking through

PKA- and PKC-dependent phosphorylation signaling mechanisms (Tritsch and Sabatini, 2012). Rapid reversal of potentiated AMPAR signaling by cocaine re-exposure might reflect engagement of D1 receptors that promote internalization of AMPARs. Consistent with this, one report demonstrated that blocking clathrin-dependent AMPAR endocytosis with a synthetic peptide prevents the induction of LTD in the NAc during abstinence from chronic amphetamine (Brebner et al., 2005). Similarly, blocking PKC-mediated phosphorylation of AMPARs attenuates reinstatement of cocaine seeking behavior (Famous et al., 2008). While more research is needed to characterize the mechanisms underlying rapid internalization of AMPARs, activation of dopamine receptors may contribute to trafficking processes that serve to promote a rapid reduction in AMPAR signaling following cocaine re-exposure.

Ex vivo cocaine engages dopamine signaling mechanisms

Here, we found that activation of DA receptors with the agonist apomorphine produced robust depotentiation of AMPAR-mediated synaptic strength, while blocking receptors with the antagonist flupenthixol prevented the *ex vivo* cocaine-induced suppression of glutamatergic synaptic strength. The ability of DA receptor signaling to bidirectionally modulate synaptic strength demonstrates that transient activation of endogenous DA signaling mechanisms is both necessary and sufficient to rapidly depotentiate elevated AMPAR synaptic transmission in the NAc shell. Notably, it is unclear exactly how cocaine augments endogenous DA in our *ex vivo* conditions, given that minimal dopamine cell firing would be expected to occur in an *ex vivo* slice

preparation. One hypothesis is that spontaneously-occurring DA release at terminals in the NAc could be enhanced by cocaine. Spontaneous DA transients mediated by individual exocytotic release events have been observed in striatal slice preparations in a number of reports (Beckstead et al., 2004; Gantz et al., 2013; Zhou et al., 2001). Since spontaneous transients occur at low frequencies and amplitudes, they may be normally undetectable in control conditions. However, acute *ex vivo* cocaine has been shown to increase the frequency and amplitude of dopamine transients in the NAc through activation of nicotinic acetylcholine receptors, increasing the ability to detect these events (Yorgason et al., 2017)—indicating a mechanism through which *ex vivo* cocaine might produce elevated synaptic dopamine in slice.

Receptor type- and cell type-specific mechanisms of dopamine function

The effects of dopamine receptor activity on AMPAR signaling in the NAc are likely to be varied and complex, given that dopamine receptor activation is capable of bidirectionally modifying excitatory synaptic strength depending on the dopamine receptor subtype and location, MSN cell subtype, level of synaptic activity, as well as previous drug experience (Tritsch and Sabatini, 2012; Wang et al., 2012). Because data from this study and previous work (Jedynak et al., 2016; Kourrich et al., 2007; Rothwell et al., 2011) were collected from pooled populations of MSNs, it is difficult to determine not only the DA receptor subtype involved but also the exact locus of the cocaine-dependent suppression of excitatory signaling. Activation of DA D1 and D2 receptors in the NAc has been shown to be necessary both for the expression of behavioral

sensitization (Henry et al., 1998; Mattingly et al., 1994) and reinstatement of cocaine seeking (Anderson et al., 2005, 2003; Schmidt et al., 2006), indicating a role for both receptor types in cocaine-related behavior and NAc function following drug re-exposure. However, a significant body of literature indicates that cocaine-related synaptic plasticity occurs preferentially at D1-expressing MSNs of the NAc (MacAskill et al., 2014; Pascoli et al., 2014, 2011; Terrier et al., 2016). Depotentiation of AMPAR signaling might therefore reflect a reversal of AMPA signaling specifically at D1-MSN synapses previously potentiated by cocaine experience.

CB1 receptors mediate reversal of enhanced AMPAR transmission in the NAc

Within the striatum, eCB signaling is a critical regulator of synaptic activity and plasticity, facilitating synaptic depression at glutamatergic synapses on MSNs through CB1 receptor-mediated suppression of excitatory transmission (Gerdeman et al., 2002; Kreitzer and Malenka, 2007; Robbe et al., 2002). Furthermore, accumulating evidence indicates a critical role for eCB signaling in reward-related behavior and facilitation of excitatory synaptic plasticity in the striatum (Wiskerke et al., 2008; Zlebnik and Cheer, 2016). Given the fundamental role that eCBs play in dampening excitatory signaling throughout the striatum, we hypothesized that eCB signaling mechanisms might be engaged by cocaine re-exposure to promote depotentiation of synaptic strength in the NAc shell. While evidence of the impact of repeated cocaine on eCB-mediated signaling and synaptic plasticity in the NAc is still relatively limited, several studies previously suggested that eCB-mediated plasticity in the striatum is impaired by acute cocaine

(Fourgeaud et al., 2004; Grueter et al., 2010). However, more recent work demonstrated that CB1 signaling remains functionally intact following repeated cocaine (McCutcheon et al., 2011; Ortinski et al., 2012). In the present study, we define a specific role for CB1 receptors in reversing enhanced NAc AMPAR transmission during abstinence from cocaine. We observed that application of the CB1 receptor antagonist/inverse agonist SR141716A prevented the cocaine-induced reduction in mEPSC frequency while partially rescuing the challenge-induced reduction in mEPSC amplitude, indicating stronger modulation of pre-synaptic release properties. Additionally, we found that activation of CB1 receptors with the agonist WIN 55,212-2 was sufficient to depotentiate both mEPSC amplitude and frequency. These findings confirm that CB1 signaling remains functionally intact following repeated cocaine and is capable of modulating mechanisms of glutamatergic synaptic plasticity in the NAc shell.

To our knowledge, our data are the first to report that CB1 signaling modifies post-synaptic AMPAR signaling during protracted abstinence from cocaine. An issue of contention regarding eCB-mediated synaptic plasticity has been whether activation of CB1 receptors alone is sufficient to induce depression (i.e. eCB-LTD) (Kreitzer and Malenka, 2005). The ability of the CB1 agonist WIN 55,212-2 to suppress both mEPSC frequency and amplitude in the present study is somewhat surprising given the pre-synaptic localization of these receptors on glutamatergic afferents in the NAc. However, there is some evidence that WIN 55,212-2 is capable of robustly depressing measures of pre-synaptic and post-synaptic glutamatergic transmission in the striatum. One report observed that activation of CB1 receptors with WIN 55,212-2 (using the same

concentration as this study) is sufficient to depress electrically-evoked EPSCs in striatal MSNs, an effect that did not require post-synaptic GPCR signaling (Kreitzer and Malenka, 2005). Similarly, CB1 activation with WIN 55,212-2 depressed both mEPSC amplitude and frequency in the NAc, indicating post-synaptic modulation of AMPAR currents (Hoffman and Lupica, 2001). Additionally, endogenous and exogenous cannabinoid ligands have been shown to alter post-synaptic signaling properties by modulating several ionic conductances (Deadwyler et al., 1995; Mackie et al., 1995; Schweitzer, 2000) or other non-CB1 receptors located on the post-synaptic membrane including TRPV1 receptors, peroxisome proliferator-activated receptors (PPARs), and G-protein receptor 55 (GPR55) and 119 (GPR119) (Grueter et al., 2010; Parsons and Hurd, 2015). However, it is important to note that the majority of these studies examined CB1-mediated synaptic depression under basal (non-drug) conditions; it is still relatively unknown how CB1 signaling engages and potentially alters cellular mechanisms of plasticity at synapses altered by chronic cocaine exposure. It seems likely that the mechanisms required to suppress excitatory signaling would be different at synapses under altered glutamate homeostasis, as is the case following chronic cocaine exposure (Knackstedt and Kalivas, 2009). Additional studies will be needed to elucidate how CB1 receptor activation is capable of broadly modulating both pre- and post-synaptic glutamatergic transmission in the NAc following experience with cocaine.

We observed that application of the CB1 antagonist SR141716A caused a moderate (but not significant) elevation in AMPAR transmission in MSNs from mice pre-treated with saline or cocaine but not challenged with cocaine *ex vivo*. This could be

evidence for SR141716A acting as an inverse agonist, relieving CB1-mediated inhibition of glutamate release exerted by reducing the activity of constitutively-active CB1 receptors. Alternatively, this could indicate antagonism of CB1 activity through suppression of tonic eCB signaling. While early studies characterized SR141716A as an inverse agonist (Bouaboula et al., 1997; Maneuf and Brotchie, 1997; Rinaldi-Carmona et al., 1994), these studies were conducted in heterologous expression systems or dissociated cell cultures—reduced systems where tonic levels of eCBs are extremely low compared to physiological brain levels (Pan et al., 2008; Pertwee, 2005). Furthermore, studies examining glutamatergic synaptic plasticity in the striatum have found that SR141716A does not consistently affect basal synaptic transmission (Auclair et al., 2000; Huang et al., 2001) and, more importantly, fully reverses WIN 55,212-2-mediated synaptic depression (Gerdeman and Lovinger, 2001; Hoffman and Lupica, 2001; Robbe et al., 2001, 2002). Therefore, it seems likely that the inverse agonism effects of SR141716A under these experimental conditions are minimal at best, and that this compound is acting chiefly as an antagonist.

Potential cellular mechanisms of eCB-mediated synaptic depotentiation

Given that striatal CB1 signaling plays a central role in synaptic depression at glutamatergic synapses, our data suggest that cocaine re-exposure during abstinence engages similar CB1 signaling mechanisms that promote a reduction in glutamatergic synaptic transmission at NAc MSN synapses. CB1 signaling in the NAc therefore might participate in reducing excitatory synaptic strength by interacting with a number of

potential cellular mechanisms. A substantial body of evidence has demonstrated that eCB-mediated depression of glutamatergic transmission in the striatum commonly requires activation of Group I mGluRs (mGluR1 and mGluR5) (Lüscher and Huber, 2010). Activation of post-synaptic mGluR1/5 on NAc MSNs promotes the release of eCBs, which activate pre-synaptic CB1 receptors to decrease neurotransmitter release probability and promote synaptic depression (Castillo et al., 2012; Robbe et al., 2002). Enhanced glutamatergic synaptic transmission in the NAc elicited by re-exposure to cocaine (McFarland et al., 2003; Park et al., 2002; Xi et al., 2006) may therefore activate mGluR1/5 and initiate eCB signaling, promoting a reduction in synaptic strength. Previously, our lab demonstrated that mGluR5 signaling in the NAc shell is necessary for cocaine-induced AMPAR synaptic depotentiation (Chapter 1). These findings showing that synaptic depotentiation requires CB1 receptor activity in the NAc shell could reflect serial activation of mGluR5 and CB1 receptors that participate in reducing glutamatergic synaptic transmission in the NAc shell following cocaine re-exposure.

In addition, dopamine D2 receptors have been shown to cooperate with Group I mGluRs to promote eCB signaling and excitatory synaptic depression in the striatum (Kreitzer and Malenka, 2005; Yin and Lovinger, 2006). Glutamatergic synapses onto D2 receptor-expressing MSNs in the striatum preferentially express eCB-mediated LTD compared to D1-MSNs (Grueter et al., 2010; Kreitzer and Malenka, 2007), and several studies have reported a requirement for D2 receptor activation in striatal eCB-LTD (Kreitzer and Malenka, 2007; Shen et al., 2008; Wang et al., 2006). A potential explanation for this phenomenon is that D2 receptor activation facilitates eCB production

in the striatum (Centonze et al., 2004; Giuffrida et al., 1999; Patel et al., 2003; Wang et al., 2006), and may cooperate with Group I mGluRs to increase synthesis of eCBs to promote synaptic depression. Increased synaptic availability of both glutamate and dopamine due to cocaine re-exposure may therefore activate mGluR5 and D2 receptors that work together to dampen excitatory synaptic strength. While additional studies are needed to determine the cell type- and receptor type-specific mechanisms by which cocaine re-exposure engages both dopamine and eCB signaling mechanisms to modify glutamatergic synaptic plasticity, our findings demonstrate that the eCB system, a central mechanism mediating synaptic depression in the striatum, participates in attenuating potentiated AMPAR synaptic transmission during abstinence from cocaine. Given that increasing clinical evidence suggests that an interaction between both the dopamine and eCB systems is involved in the pathophysiology of numerous disorders involving the striatum (García et al., 2016; Kuepper et al., 2010; Parolaro and Rubino, 2008; Pisani et al., 2011), understanding the complex interactions between these two systems may be critical in developing future therapeutic strategies for relapse.

Chapter 4: Cocaine-evoked synaptic plasticity is MSN cell type-specific and is modulated by distinct receptor types

Introduction

In the striatum, two distinct MSN populations may be distinguished based on their selective expression of dopamine D1 receptors (D1-MSNs) or dopamine D2 receptors (D2-MSNs). Furthermore, these cell populations differ in their downstream projection targets. While D1-MSNs project directly to the midbrain ("direct pathway"), D2-MSNs take a multi-synaptic route through pallidal nuclei before synapsing on midbrain targets ("indirect pathway"). Strictly in terms of motor output, D1-MSNs facilitate activation of motor systems while D2-MSNs suppress output. Given the coupling of differential dopamine receptors to these cell types as well as their divergent projections to downstream motor circuitry governing behavior, recent literature in the field has sought to characterize how cocaine-induced adaptations at synapses on specific MSN cell sub-types might underlie long-lasting relapse vulnerability.

Emerging evidence demonstrates that D1- and D2-MSNs mediate distinct, and often opposing, roles for reward-related learning and reinforcement. For example, optogenetic stimulation of D1-MSNs *in vivo* produces reinforcement learning in mice, while activation of D2-MSNs suppresses behavior (Kravitz et al., 2012). In addiction-related studies, D1-MSNs seem to play a more dominant role in producing the rewarding and reinforcing effects of drugs of abuse, with more robust molecular, cellular, and synaptic alterations occurring in D1-MSNs (Lobo and Nestler, 2011). In a cocaine conditioned place preference paradigm, activation of D1-MSNs in the NAc enhances the

rewarding effects of cocaine, while D2-MSN stimulation suppresses reward (Lobo et al., 2010), an effect that involves cellular signaling mechanisms distinct between the two cell sub-types.

Numerous studies have demonstrated that cocaine-induced changes in glutamatergic synaptic strength occur almost exclusively at D1-MSN synapses (Kim et al., 2011; MacAskill et al., 2014; Terrier et al., 2016; Pascoli et al., 2011, 2014), suggesting that D1-MSNs mediate persistent excitatory plasticity induced by repeated or chronic drug exposure. However, it is not known whether reductions in enhanced AMPAR transmission elicited by renewed cocaine exposure occur exclusively at synapses on a particular MSN sub-type. Furthermore, the cellular mechanisms of plasticity at synapses on D1- and D2-MSNs have not been elucidated. The purpose of the following experiments therefore was two-fold: 1) Characterize cocaine-induced AMPAR plasticity in specific MSN sub-types in the NAc shell; 2) Investigate cellular mechanisms mediating AMPAR plasticity at specific MSN synapses in the NAc shell.

Materials and methods

Animals

Adult male and female mice (P48-60) were heterozygous BAC transgenic mice, in which expression of the fluorescent proteins tdTomato and eGFP were driven by D1 (drd1a-tdtomato) or D2 (drd2-eGFP) dopamine receptors, respectively. All animals were group-housed in a temperature- and humidity-controlled environment on a 12 hr light/12

hr dark cycle, with food and water available *ad libitum.* All experiments were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Behavioral sensitization

Prior to testing, mice were habituated to experimenter handling, *i.p*. injections, and the testing environment (individual activity boxes, 8.5 x 17.5 x 9 in) over two days. On five consecutive testing days, mice were habituated to the testing environment for 30 minutes (min) before receiving an intraperitoneal injection of either cocaine (15 mg/kg) or saline. Mice were immediately placed back into the testing environment, and their activity was monitored for 90 min using a video-based tracking system (Any-Maze, Stoelting, WI, USA). Animals were returned to their home cages at the end of each testing period. Following the last day of testing, animals remained in their home cages in the colony for 10 – 14 days before electrophysiology experiments were performed. To determine whether repeated cocaine treatment produced behavioral sensitization, the locomotor activity (distance traveled) during the first 30 minutes of the session on Day 1 and Day 5 was compared, pooling all cocaine-treated animals. A t-test was used to confirm a significant difference in locomotor activity between Day 1 and Day 5, indicating the development of behavioral sensitization to cocaine. Animals that did not fit these criteria were excluded from recording experiments.

2.3 Slice electrophysiology

Following 10–14 days of abstinence from cocaine treatment, mice were anesthetized with isoflurane, decapitated, and the brain rapidly removed. Parasagittal slices (240 µm) containing the nucleus accumbens shell were prepared in 2-4°C sucrose-containing artificial cerebrospinal fluid (ACSF), after which slices rested for at least 30 min in standard ACSF at room temperature. To assess AMPAR-mediated excitatory synaptic transmission, cells were voltage-clamped at -80 mV using an Axon Instruments MultiClamp 700A (Molecular Devices, Sunnyvale, CA, USA). Data were filtered at 2 kHz, digitized at 5 kHz, and collected using custom Igor Pro software (Wavemetrics, Lake Oswego, OR, USA). MiniAnalysis software (Synaptosoft, Decatur, GA, USA) was used offline to analyze mEPSC amplitude and frequency. For a more detailed description of slice preparation and recording methods, see Chapter 1.

2.4 *Ex vivo drug application*

Drugs were prepared in a stock solution and added at the final concentration to the ACSF perfusing the slices. A between-cell experimental design was utilized in which slices were bathed in the drug or control ACSF prior to recording synaptic activity. During these experiments, slices were exposed to one of the following conditions:

1. No challenge (exposure to ACSF only)
2. *Ex vivo* cocaine challenge (10 µM; 10 min)
3. *Ex vivo* SCH23390 (D1 antagonist; 3µM) *or* sulpiride (D2 antagonist; 10 µM) alone (10 min) + in the presence of 10 µM cocaine (10 min)
4. *Ex vivo* SKF81297 (D1 agonist; 20 µM) *or* quinpirole (D2 agonist; 10 µM) alone

5. *Ex vivo* SR141716A (CB1 antagonist, 1µM) alone (10 min) + in the presence of 10 µM
6. *Ex vivo* SR141716A (CB1 antagonist; 1µM) alone (10 min) + in the presence of SKF81297 *or* quinpirole (D1 or D2 agonist)

Following the timed *ex vivo* drug exposure(s), the perfusion was switched back to standard ACSF for recording. Recordings took place up to 1 hour following *ex vivo* drug exposure.

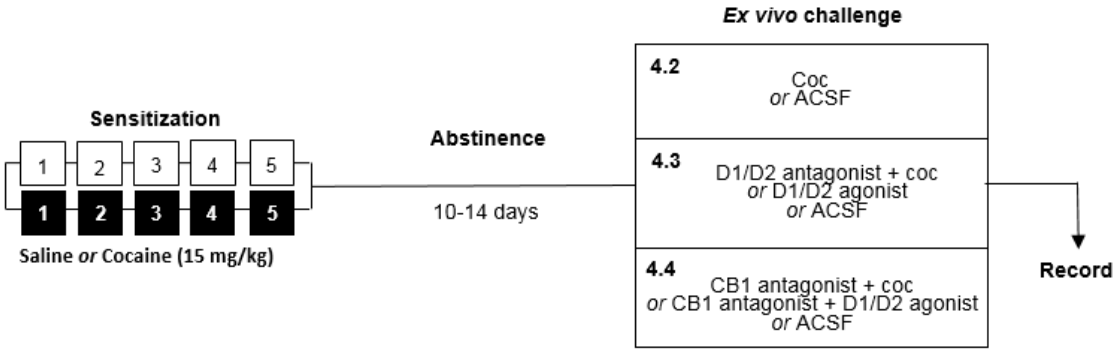

Figure 4.1. Timeline of experimental manipulations. Mice received five once-daily injections of cocaine or saline, followed by 10-14 days of abstinence in their home cages. To characterize cocaine-induced synaptic depotentiation in specific MSNs, brain slices were prepared following abstinence and bath applied with *ex vivo* cocaine (10 µM). Next, to study the role of specific dopamine receptors on MSNs, a D1 *or* D2 antagonist (SCH23390 or sulpiride) was added to the bath prior to cocaine challenge; or, a D1 *or* D2 agonist (SKF 81297; quinpirole) was substituted in place of cocaine. Lastly, to examine DA-CB1 signaling interactions, slices were first exposed to a CB1 antagonist (SR141716A) prior to cocaine challenge; or, slices were exposed to the CB1 antagonist prior to incubation in the D1 *or* D2 agonist.

2.5 Drugs

SCH23390, SKF81297, sulpiride, quinpirole, and SR141716A were purchased from Tocris Bioscience (Bristol, United Kingdom). Lidocaine and picrotoxin were purchased from Sigma Aldrich (St. Louis, MO, USA). Cocaine was obtained from

Boynton Health Services Pharmacy (University of Minnesota, Minneapolis, MN, USA). Drugs were prepared in a stock solution at 100 – 1000 times the desired concentration and added into standard ACSF at the final concentration. SR141716A stock was dissolved in DMSO, with the final concentrations of DMSO <0.01%.

2.5 Statistical analysis

All data are presented as group mean ± SEM. Statistical significance was assessed with a Student's t-test, one-way ANOVA, or two-way ANOVA using JMP Pro (SAS, Cary, NC, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Tukey HSD *post hoc* tests were used for pairwise comparisons where appropriate. The threshold for significance was $p<0.05$.

Results

Cocaine-induced changes in NAc shell AMPAR synaptic strength are MSN type-specific.

In the NAc, MSNs may be segregated based on their selective expression of dopamine D1-type receptor (D1-MSNs) or D2-type receptor (D2-MSN). These distinct cell subpopulations project to different downstream targets and have been shown to oppositely regulate reward-related behavior (Lobo and Nestler, 2011). Furthermore, plasticity at excitatory synapses in the NAc is differentially altered by experience with cocaine. Several studies have demonstrated that cocaine-induced changes in glutamatergic signaling occur almost exclusively at D1-MSN synapses (Kim et al., 2011; Lobo and Nestler, 2011; Pascoli et al., 2011; Terrier et al., 2016). However, while these

studies characterized alterations in synaptic transmission that occur following repeated or chronic cocaine administration, it is not yet clear whether subsequent cocaine re-exposure during abstinence reduces AMPAR signaling in the NAc in a cell type-specific manner. To investigate cell-type specific synaptic depotentiation, we performed recordings of mEPSCs following cocaine *ex vivo* challenge during abstinence from cocaine (10-14 days). We found that in D1-MSNs, abstinence from repeated cocaine potentiated mEPSC amplitude (picoamperes of current, *pA*), which was reversed by *ex vivo* cocaine challenge [**figure 4.2C**; Sal (9.93 ± 0.37, 7 cells/5 animals); Coc (14.60 ± 1.14, 8 cells/6 animals); Coc-coc (9.60 ± 30, 7 cells/6 animals); $F_{2,20}$=12.37, p=0.0003]. Similarly, mEPSC frequency (events per second, *hz*) was increased during abstinence but reversed by *ex vivo* cocaine [**figure 4.2C**; Sal (3.59 ± 0.75); Coc (8.57 ± 1.28); Coc-coc (2.47 ± 0.20); $F_{2,20}$=12.08, p=0.0004]. However, cocaine re-exposure had no effect on mEPSC amplitude in D2-MSNs [**figure 4.2D**; Sal (10.69 ±84, 4 cells/3 animals); Coc (9.74 ± 0.55, 9 cells/8 animals); Coc-coc (10.73 ± 0.52, 8 cells/7 animals); $F_{2,19}$=0.96; p=0.4013]. Interestingly, cocaine challenge produced a modest increase in mEPSC frequency in D2-MSNs relative to saline treated animals [Sal (2.78 ± 0.07); Coc (3.78 ± 0.39); Coc-coc (5.48 ± 0.80); $F_{2,19}$=4.022, p=0.035], suggesting modulation of pre-synaptic mechanisms of plasticity in D2-MSNs.

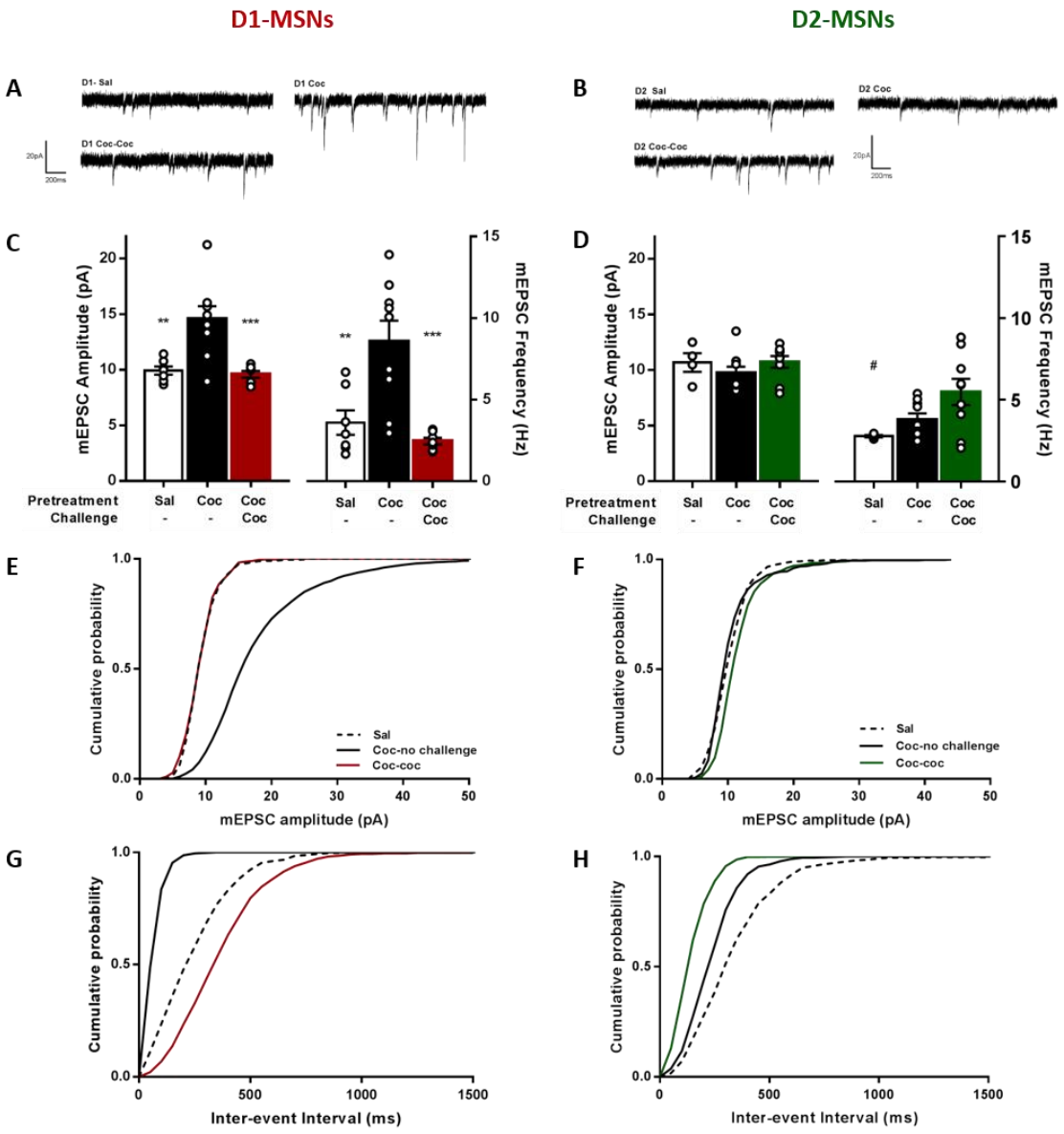

Figure 4.2. Cocaine-induced changes in NAc shell AMPAR synaptic strength are MSN type-specific. (A,B) Representative mEPSC current traces from NAc shell D1-MSNs and D2-MSNs. (C) Mean mEPSC amplitude and frequency in NAc shell D1-MSNs from saline + no challenge (Sal), cocaine + no challenge (Coc), and cocaine + *ex vivo* cocaine challenge (Coc). (D) Mean mEPSC amplitude and frequency in NAc shell D2-MSNs from saline + no challenge (Sal), cocaine + no challenge (Coc), and cocaine + *ex vivo* cocaine challenge (Coc). (E,G) Cumulative probability distributions of mEPSC amplitude and inter-event interval in D1-MSNs from saline + no challenge (Sal), cocaine + no challenge (Coc), cocaine + *ex vivo* cocaine challenge (Coc-coc). (F, H) Cumulative probability distributions of mEPSC amplitude and inter-event interval in D2-MSNs from saline + no challenge (Sal), cocaine + no challenge (Coc), cocaine + *ex vivo* cocaine

challenge (Coc-coc). All data are presented as mean ± SEM. * $p<0.05$ vs. Coc, ** $p<0.01$ vs Coc, ***$p<0.001$ vs. Coc; # $p<0.05$ vs. Coc-coc.

Cell type-specific bidirectional plasticity in the NAc shell is differentially modulated by dopamine receptors.

Next, we asked whether specific dopamine receptors on D1-MSNs and D2-MSNs mediate AMPAR synaptic depotentiation. A major effect of cocaine is to elevate synaptic DA levels, leading to activation of DA receptors. DA receptor activity has been shown to alter AMPAR signaling at glutamatergic inputs on NAc MSNs (Calabresi et al., 2007; Wolf and Ferrario, 2010). Notably, D1 receptors are metabotropic receptors that promote increased production of cAMP and PKA phosphorylation through their coupling to G_s proteins, and have been demonstrated to modify the trafficking of AMPARs at the synapse through phosphorylation of AMPAR subunits (Chao et al., 2002; Mangiavacchi and Wolf, 2004) . However, it is not clear how D1 receptor activity modulates changes in AMPAR synaptic transmission following cocaine experience. Furthermore, D2 receptors have been shown to reduce AMPAR-transmission in basal conditions (André *et al*, 2010; Hernández-Echeagaray *et al*, 2004) and following cocaine challenge (Ferrario et al., 2011), raising the possibility that D2 activation might mediate an overall reduction in AMPAR synaptic strength following cocaine re-exposure. Bath application of the D1 receptor-specific antagonist SCH23390 (3 μM) prior to *ex vivo* cocaine challenge blocked the induction of the depotentiation of mEPSC amplitude in D1-MSNs (**figure 4.3C**; 14.44 ± 0.90, 7 cells/6 animals; $F_{4,30}=9.26$, $p<0.0001$) and frequency (**figure 4.3C**; 5.66 ± 1.39; $F_{4,30}=8.85$, $p<0.0001$), while sulpiride (D2 antagonist; 10 μM) had no effect on

mEPSC amplitude (**figure 4.3D**; 9.88 ± 0.36, 4 cells/4 animals; p=0.09) or frequency (**figure 4.3D**; 3.32 ± 0.73, p=0.98) in D2-MSNs.

These data thus far demonstrate that dopamine D1 receptor signaling at synapses on D1-MSNs is necessary for cocaine-induced reversal of AMPAR signaling. Next, we asked whether dopamine receptor stimulation is sufficient to induce depotentiation. Application of the D1 receptor agonist SKF 81297 (20 μM) produced a reduction in mEPSC amplitude (**figure 4.3C**; 11.47 ± 0.52, 6 cells/3 animals; p=0.048) and frequency (**figure 4.3C**; 3.58 ± 0.68; p=0.0018) in D1-MSNs, while the D2 receptor agonist quinpirole (10 μM) had no effect on mEPSC amplitude (**figure 4.3D;** 10.17 ± 0.84, 5 cells/2 animals; p=0.99). However, application of quinpirole produced very low mEPSC frequency in D2-MSNs (**figure 4.3D**; 1.55 ± 0.25). Likely due to the high variability of sample observations in this dataset, this finding was not significantly different from mEPSC frequency in saline pre-treated animals (Sal) or cocaine pre-treated animals before challenge (Coc); however, it did differ significantly from cells treated with *ex vivo* cocaine (Coc-coc).

In a limited set of control experiments, application of the D1 antagonist SCH23390 during *ex vivo* cocaine re-exposure did not alter synaptic transmission in D2-MSNs from animals in cocaine abstinence [amplitude: 10.85 ± 0.14, p=0.6760; frequency: 3.43 ± 0.56, p=0.995; 5 cells/4 animals; data not shown], and similarly, the D1 agonist SKF81297 did not alter transmission in D2-MSNs compared to slices from animals in abstinence (amplitude: 9.68 ± 0.76, p>0.99; frequency: 4.45 ± 0.80, p=0.9620; data not shown). Similarly, the D2 antagonist sulpiride in the presence of cocaine did not

modify synaptic strength in D1-MSNs during abstinence (amplitude: 13.48 ± 0.36, p=0.9483; frequency: 4.20 ± 0.49, p=0.1163; 3 cells/1 animal, data not shown), and application of the D2 agonist quinpirole furthermore did not alter D1-MSN transmission compared to slices from animals in abstinence (amplitude: 16.42 ± 2.80, p=0.8405; frequency: 5.80 ± 2.10, p=0.6504; 2 cells/1 animal, data not shown). These findings indicate no evidence for cross-modulation of DA receptor signaling in specific MSN cell types, with no effect of D2 receptor modulation observed in MSNs expressing D1 receptors, and similarly no effect of D1 modulation in D2-expressing MSNs. Thus, the ability of a particular DA receptor type to modulate synaptic transmission is specific to MSNs expressing that specific receptor type.

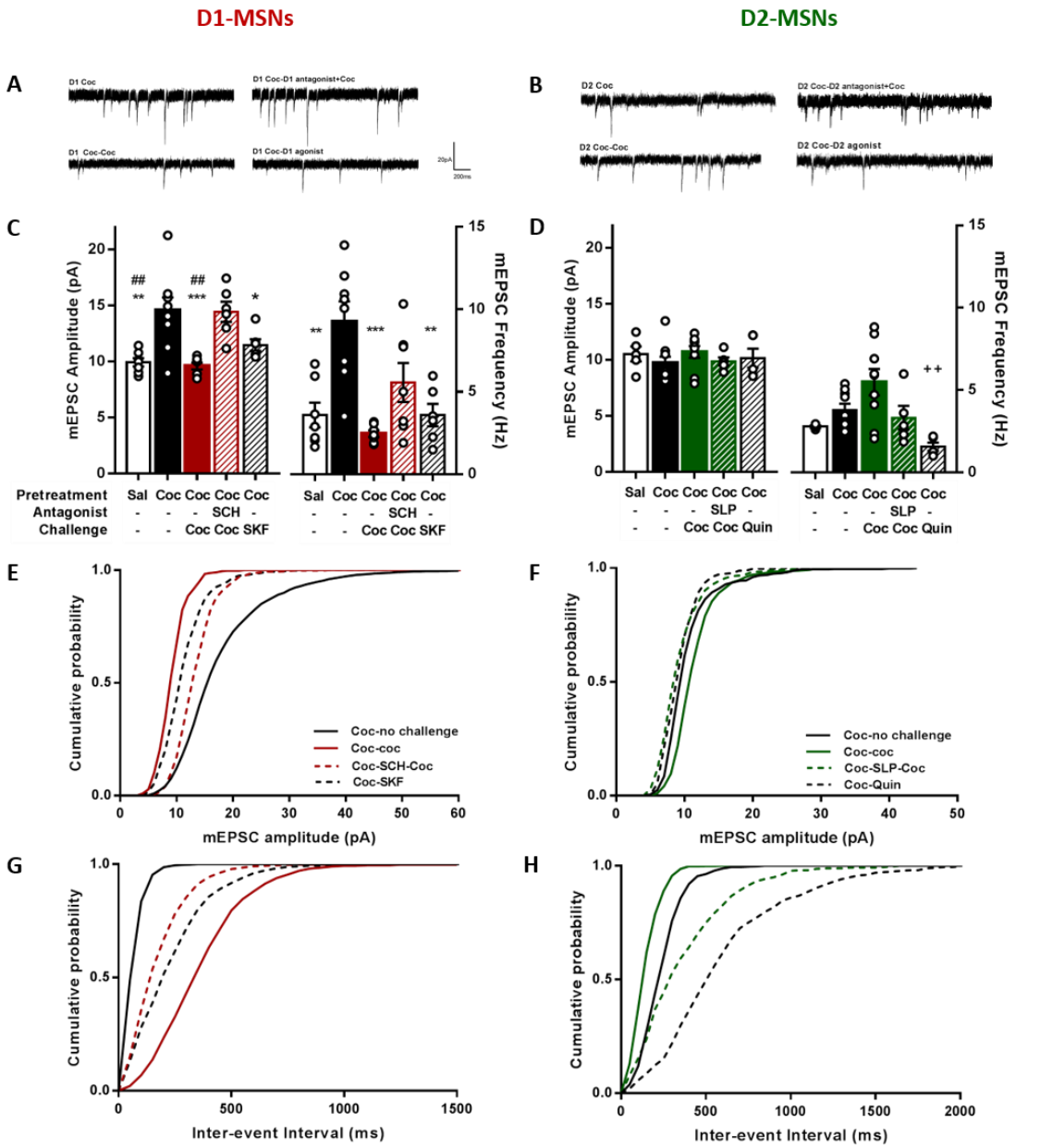

Figure 4.3. Cell type-specific bidirectional plasticity in the NAc shell is differentially modulated by dopamine receptors. (A,B) Representative mEPSC traces from NAc shell D1-MSNs and D2-MSNs. (B) Mean mEPSC amplitude and frequency in NAc shell D1-MSNs from saline + no challenge (Sal), cocaine + no challenge (Coc), cocaine + *ex vivo* cocaine challenge (Coc-coc), cocaine + SCH23390/coc (Coc-SCH-Coc), and cocaine + SKF81297 (Coc-SKF). (C) Mean mEPSC amplitude and frequency in NAc shell D2-MSNs from saline + no challenge (Sal), cocaine + no challenge (Coc), cocaine + *ex vivo* cocaine challenge (Coc-coc), cocaine + sulpiride/coc (Coc-SLP-Coc), and cocaine + quinpirole (Coc-Quin). (E,G) Cumulative probability distributions of mEPSC amplitude and inter-event interval in D1-MSNs from cocaine + no challenge (Coc), cocaine + *ex vivo* cocaine challenge (Coc-coc), cocaine + SCH23390/coc

(Coc-SCH-Coc), and cocaine + SKF81297 (Coc-SKF). (F,H) Cumulative probability distributions of mEPSC amplitude and inter-event interval in D2-MSNs from cocaine + no challenge (Coc), cocaine + *ex vivo* cocaine challenge (Coc-coc), cocaine + sulpiride/coc (Coc-SLP-Coc), and cocaine + quinpirole (Coc-Quin). All data are presented as mean ± SEM. * $p<0.05$ vs. Coc, ** $p\leq0.01$ vs. Coc, *** $p\leq0.001$ vs Coc; # $p<0.05$ vs. Coc-SCH-Coc; ## $p<0.01$ vs. Coc-SCH-Coc; ++$p<0.001$ vs. Coc-coc.

Dopamine and endocannabinoid modulation of cocaine-induced AMPAR plasticity in the NAc shell is cell type-specific.

In addition to the known role of dopamine in modulating excitatory synaptic plasticity in the striatum, endogenous cannabinoids (eCBs) prominently shape synaptic activity and plasticity in the striatum (Gerdeman et al., 2002; Robbe et al., 2002). Previous studies indicate that eCB-mediated synaptic plasticity requires D2 receptor activation and furthermore occurs selectively in D2-expressing MSNs of the striatum. However, little is known regarding how cocaine experience alters neuromodulatory signaling at excitatory synapses on specific MSN cell subtypes. To investigate this, we first examined the role of CB1 signaling in synaptic depotentiation within specific MSN cell sub-types. In D1-MSNs, bath application of the CB1 receptor antagonist SR141716A (1 μM) produced a modest blockade of the depotentiation of mEPSC amplitude (**figure 4.4C**; 12.6 ± 0.52, 6 cells/3 animals; $F_{5,35}=7.891$, $p<0.0001$) and a more robust blockade of the reduction in mEPSC frequency (**figure 4.4C**; 5.51 ± 0.84; $F_{5,34}=7.76$, $p<0.0001$). In D2-MSNs, SR141716A had no effect on mEPSC amplitude (**figure 4.4D**; 9.82 ± 0.40, 7 cells/4 animals; $F_{5,33}=0.46$, $p=0.80$) or frequency (**figure 4.4D**; 3.09 ± 0.66; $p=0.96$).

Several lines of evidence suggest that in the striatum, activation of dopamine receptors facilitates eCB production that participates in modifying synaptic efficacy. A number of studies have demonstrated that under basal (non-drug) conditions, activation

of dopamine D2 receptors in the striatum (dorsal and ventral) is sufficient to promote increased production of eCBs (Centonze et al., 2004; Giuffrida et al., 1999; Wang et al., 2006). However, it is unknown whether these mechanisms play a role in reducing synaptic strength following cocaine exposure. Therefore, we asked whether DA receptor-mediated depotentiation of AMPAR signaling requires serial activation of CB1 receptors as well. Pre-incubation of slices in the CB1 receptor antagonist SR141716A prior to bath application of the D1 receptor agonist SKF 81297 (20 μM) in D1-MSNs produced depotentiation of mEPSC amplitude (**figure 4.4C**: 10.33 ± 0.52, 6 cells/4 animals; p=0.002) but blocked the reduction of mEPSC frequency (**figure 4.4C**; 5.39 ± 1.28, p=0.049). In D2-MSNs, mEPSC amplitude was unaffected by exposure to the CB1 antagonist in the presence of the D2 receptor agonist quinpirole (**figure 4.4D**; 10.32 ± 0.82, 6 cells/3 animals; p=0.8009); however, while mEPSC frequency was not significantly changed relative to saline pre-treated animals (Sal) or cocaine pre-treated animals (Coc), bath exposure to the CB1 antagonist during incubation in the D2 agonist produced a visible elevation in frequency relative to the quinpirole-mediated suppression in D2-MSNs (**figure 4.4D**; 4.56 ± 0.80; p=0.0581). Nevertheless, this tendency toward an increase in frequency was not significantly different compared to other groups.

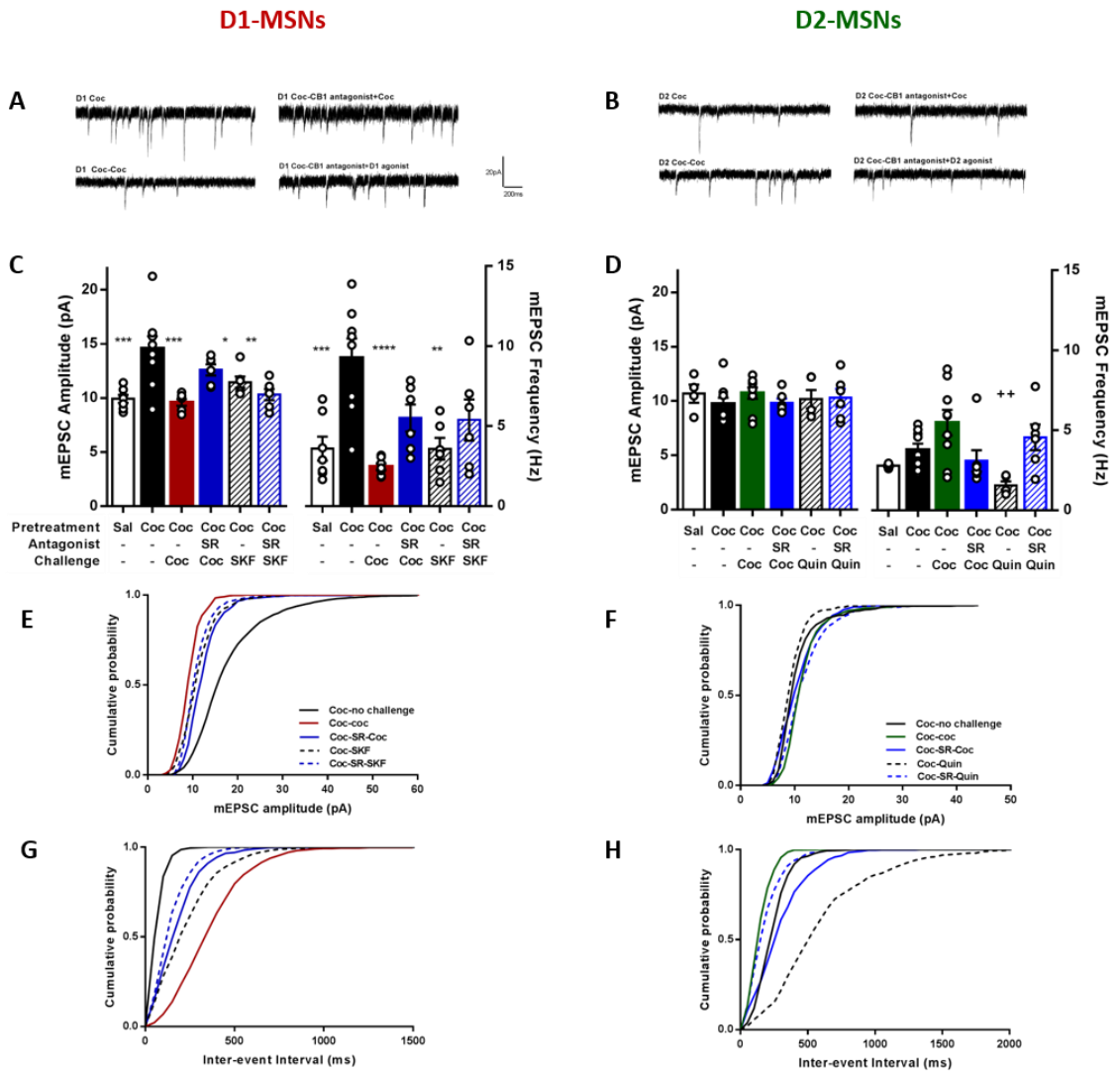

Figure 4.4. Dopamine and endocannabinoid modulation of cocaine-induced AMPAR plasticity in the NAc shell is cell type-specific. (A,B) Representative mEPSC traces from NAc shell D1-MSNs and D2-MSNs. (B) Mean mEPSC amplitude and frequency in NAc shell D1-MSNs from saline + no challenge (Sal), cocaine + no challenge (Coc), cocaine + *ex vivo* cocaine challenge (Coc-coc), cocaine + SR141716A/coc (Coc-SR-Coc), cocaine + SKF81297 (Coc-SKF), and cocaine + SR141716A/SKF81297 (Coc-SR-SKF). (C) Mean mEPSC amplitude and frequency in NAc shell D2-MSNs from saline + no challenge (Sal), cocaine + no challenge (Coc), cocaine + *ex vivo* cocaine challenge (Coc-coc), cocaine + SR141716A/coc (Coc-SR-Coc), cocaine + quinpirole (Coc-Quin), and cocaine + SR141716A/quinpirole (Coc-SR-Quin). (E,G) Cumulative probability distributions of mEPSC amplitude and inter-event interval in D1-MSNs from cocaine + no challenge (Coc), cocaine + *ex vivo* cocaine challenge (Coc-coc), cocaine + SR141716A/coc (Coc-SR-Coc), cocaine + SKF81297 (Coc-SKF), and cocaine + SR141716A/SKF81297 (Coc-SR-SKF). (F,H) Cumulative probability distributions of mEPSC amplitude and inter-event interval in D2-MSNs from cocaine + no challenge (Coc), cocaine + *ex*

vivo cocaine challenge (Coc-coc), cocaine + SR141716A/coc (Coc-SR-Coc), cocaine + quinpirole (Coc-Quin), and cocaine + SR141716A/quinpirole (Coc-SR-Quin). All data are presented as mean ± SEM. * $p<0.05$ vs. Coc, ** $p\leq0.01$ vs. Coc, *** $p\leq0.001$ vs Coc, **** $p<0.0001$ vs. Coc; ++$p<0.01$ vs. Coc-Coc.

Discussion

Within the striatum, repeated cocaine exposure causes enduring adaptations in glutamatergic synaptic strength in NAc MSNs. To date, substantial evidence demonstrates that cocaine appears to selectively alter excitatory synaptic transmission at synapses on D1-expressing MSNs (Kim et al., 2011; Pascoli et al., 2014, 2011; Terrier et al., 2016), neurons of the striatal "direct pathway" that have been shown to critically mediate reward-related learning and approach behavior (Lobo and Nestler, 2011). However, it is currently unknown whether the reversal of enhanced AMPAR synaptic transmission induced by cocaine re-exposure is mediated by specific MSN cell types in the NAc shell. One would predict that synaptic adaptations occurring selectively on D1-MSNs would likely be reversed on D1-MSNs as well; yet, it is possible that depotentiation of synaptic strength could reflect a reduction in basal synaptic transmission in D2-MSNs. Given the opposing roles these cell types appear to exert on reward-seeking behavior, such a reduction in signaling at D2-MSNs might produce a subsequent shift in excitatory drive toward D1-MSN activation, promoting renewed drug-seeking behavior.

Our findings, however, appeared to confirm our former hypothesis: similar to previous studies, abstinence from repeated cocaine selectively enhanced AMPAR synaptic transmission at D1-MSN synapses in the NAc shell, while cocaine re-exposure

ex vivo reversed enhanced signaling at D1-MSNs. In D2-MSNs, repeated cocaine predictably had no effect on mEPSC amplitude, a measure generally considered to reflect the number and/or function of post-synaptic AMPARs. However, *ex vivo* cocaine challenge produced a noticeable elevation in the frequency of mEPSC events in D2-MSN, hinting at increased glutamate release probability at synapses on D2-MSNs.

Studies examining the expression of behavioral sensitization to cocaine (Henry et al., 1998; Mattingly et al., 1994) and reinstatement of cocaine seeking behavior following a period of abstinence or extinction (Schmidt et al., 2006; Anderson et al., 2006, 2003) commonly demonstrate a requirement of both dopamine D1 and D2 receptor signaling in the NAc, indicating that cooperative action of both of these receptor types in the NAc mediates the response to cocaine re-exposure. However, D1 receptor activation has been shown to specifically modulate alterations in NAc excitatory synaptic transmission induced by cocaine experience, while D2 signaling appears to have a minimal effect on synaptic transmission. For example, stimulation of D1 receptors during late abstinence from cocaine (3-4 weeks) is sufficient to normalize enhanced AMPAR synaptic strength in the NAc shell (Ortinski et al., 2012). Here, we observed that D1 receptor activity is both necessary and sufficient to reverse potentiated mEPSC amplitude and frequency in NAc shell D1-MSNs only, suggesting that synaptic depotentiation involves engagement of specific D1 receptors on specific D1-expressing MSNs. However, while D2 activation had no effect on mEPSC amplitude in D2-MSNs, we observed more complex effects on mEPSC frequency. Although statistically insignificant perhaps due to high variability in our sample observations, we found that a D2 receptor antagonist applied during cocaine

re-exposure appeared to rescue the increase in frequency induced by *ex vivo* cocaine in D2-MSNs, additionally reducing the variability of responses in this cell type. While this trend was apparent but not significant, further research is needed to determine how D2 signaling modulates augmented glutamate release properties following cocaine re-exposure. Additionally, application of the D2 agonist quinpirole was sufficient to suppress mEPSC frequency below basal levels, a finding which may reflect activation of pre-synaptic D2 autoreceptors that suppress excitatory signaling in the NAc (André et al., 2010; Wang et al., 2012).

These data present a rather complex and incomplete picture of how dopamine receptor activation differentially alters cocaine-mediated synaptic plasticity in the NAc. However, this study suggests that distinct receptor subtypes mediate very different forms of plasticity at NAc MSN synapses following cocaine exposure. D1 signaling appears to modify post-synaptic AMPAR signaling due to its ability to independently reverse and induce synaptic depotentiation of AMPAR mEPSC amplitude and frequency, while D2 activity modulates alterations in mEPSC frequency induced by cocaine exposure, indicating complex modulation of pre-synaptic glutamate release properties.

In the NAc, endocannabinoid signaling potently regulates excitatory synaptic transmission, mediating several forms of synaptic depression at glutamatergic synapses on NAc MSNs (Zlebnik and Cheer, 2016). Furthermore, it has been suggested that eCB-mediated synaptic depression requires activation of post-synaptic D2 receptors, occurring preferentially in D2-MSNs (Grueter et al., 2010; Kreitzer and Malenka, 2007; Shen et al., 2008). However, this has been challenged by studies demonstrating that eCB signaling is

capable of altering excitatory signaling at both D1- and D2-MSN synapses (Bagetta et al., 2011; Wang et al., 2006). A potential explanation for the D2 requirement in eCB signaling is that D2 receptor activation facilitates eCB production in the striatum (Centonze et al., 2004; Giuffrida et al., 1999; Patel et al., 2003; Wang et al., 2006), and therefore may be sufficient to reduce synaptic strength at activated synapses alone or in combination with group I mGluRs, which mobilize eCBs through G_q-mediated signaling.

Here, we found that the CB1 receptor antagonist SR141716A prevented the reduction of mEPSC amplitude and frequency induced by cocaine re-exposure in D1-MSNs. Conversely, CB1 antagonism had no effect on mEPSC amplitude in D2-MSNs but, similar to sulpiride, appeared to reverse the elevated mEPSC frequency following cocaine challenge *ex vivo.* We therefore observed that CB1 receptor activation is necessary for reducing synaptic strength at both D1- and D2-MSN synapses in the NAc. This finding is in line with recent reports contesting the notion that eCB-mediated synaptic plasticity occurs selectively at D2-MSNs. Instead, this mechanism may undergo complex modulation by other synaptic and cellular signaling factors. For example, several studies have shown that the D2 receptor dependence of striatal LTD reflects suppression of muscarinic receptor 1 (M1) signaling due to activation of D2 receptors on cholinergic interneurons, a requirement for induction of LTD at both D1- and D2-MSN synapses (Tozzi et al, 2011; Wang et al, 2006), indicating a means by which D2 receptor activity could modulate glutamatergic signaling at both synapse types.

Emerging evidence indicates that eCB and dopamine signaling mechanisms interact to modulate synaptic strength in striatal circuits (Garcia et al. 2016). To test

whether DA-mediated depotentiation of synaptic strength requires CB1 signaling, we blocked CB1 receptors while activating DA receptors. We found that the D1 agonist SKF81297 was sufficient to depotentiate mEPSC amplitude in D1-MSNs, without requiring coincident CB1 activity. However, the D1 agonist was not sufficient to reverse the potentiated mEPSC frequency, indicating that CB1 signaling downstream of D1 receptors may be necessary to dampen glutamate release from terminals on D1-MSNs. Similarly, in D2-MSNs, CB1 activation was necessary for D2 receptor-mediated suppression of mEPSC frequency, indicating that serial activation of dopamine and eCBs may be important for modulating release properties at the terminal. Potentially due to the relatively high variability of observations in this dataset, these findings in D2-MSNs were not statistically significant, but produced a visible trend. Given that different signaling mechanisms mediate plasticity at excitatory synapses on D1- vs. D2-MSNs (Lüscher and Huber, 2010), understanding how cocaine exposure drives aberrant plasticity in the NAc will require a deeper investigation of the mechanisms underlying MSN cell type-specific plasticity. These findings indicate complex modulation of pre-synaptic plasticity by dopamine and eCB signaling mechanisms.

Discussion and Conclusion

A wealth of literature has established that addiction is a pathological disorder characterized by progressive alterations in brain functions mediating reward. Drugs of abuse gradually alter brain circuits important for learning and adaptation, and drive a transition toward drug-seeking behavior. Thus, addiction may be viewed as a disorder of staged neuroplasticity, engaging mechanisms that naturally promote behavioral adaptation and plasticity, and driving maladaptive learning that motivates persistent drug-seeking (Kalivas and O'Brien, 2007; Lüscher, 2013).

The purpose of this work was to identify and characterize endogenous mechanisms of plasticity in the nucleus accumbens that are engaged by drug experience and participate in modifying synaptic function. Here, I focused on a number of neuromodulatory signaling mechanisms that are capable of finely adjusting excitatory synaptic strength in the NAc. As reviewed throughout this work, alterations in glutamatergic transmission at corticolimbic afferents to the nucleus accumbens is an important factor driving the persistence of drug-seeking behavior following chronic psychostimulant use. Coupled with this, long-term cocaine experience increases the sensitivity of MSNs to respond to glutamate input through enhanced synaptic strength mediated by upregulated AMPARs signaling. Subsequent drug re-exposure, cues associated with drug use, or stress—all common triggers for relapse—are capable of rapidly depotentiating enhanced AMPAR transmission. Understanding the neurobiological mechanisms underpinning this synaptic adaptation viewed across common relapse-related stimuli is the focus of my work here.

These studies broadly demonstrated that dopamine, endocannabinoids, and metabotropic glutamate receptors (Group I mGluRs) can have diverse effects on AMPAR-mediated synaptic function in the NAc depending on previous cocaine experience. Both dopamine and endocannabinoids, critical modulators of NAc synaptic plasticity and addiction-related behavior, contribute to cocaine-induced depotentiation of AMPAR synaptic strength, indicating that cocaine re-exposure engages multiple endogenous signaling pathways in the NAc that cooperate in modifying excitatory synaptic strength. Furthermore, we demonstrated that NAc synaptic depotentiation is rapidly induced but constrained to a relatively short time window of several (1-4) days, indicating that synapses undergo dynamic shifts in efficacy following subsequent drug re-exposures, but ultimately recover to their previously-potentiated synaptic states established by repeated, chronic drug experience. Understanding the mechanisms underlying this rapid form of plasticity could provide insight into the neurobiological correlates supporting persistent drug-seeking behavior, with the goal of prolonging abstinence by reversing or somehow compensating for these pernicious neuroadaptations.

Additionally, this work sought to characterize cellular signaling mechanisms at synapses on D1- and D2-MSNs, two distinct populations that mediate divergent responses to addicting drugs as well as differential plasticity following cocaine exposure (Smith et al., 2013). Similar to previous studies, we observed that repeated cocaine selectively potentiated synapses on D1-MSNs, while having minimal effects on D2-MSNs. Interestingly, we were more likely to find alterations in post-synaptic signaling mechanisms (i.e. mEPSC amplitude) at D1-MSN synapses, while presumed pre-synaptic

mechanisms of transmitter release (as revealed by changes in mEPSC frequency) appeared to be altered in a complex way at D2-MSNs following cocaine re-exposure. We found that in addition to the ability of D1 receptors to depotentiate AMPAR signaling at D1-MSN synapses, dopamine receptors on both cell subtypes may promote CB1 activation in a serial manner, given that CB1 receptor activation was necessary for the dopamine receptor-induced suppression of mEPSC frequency.

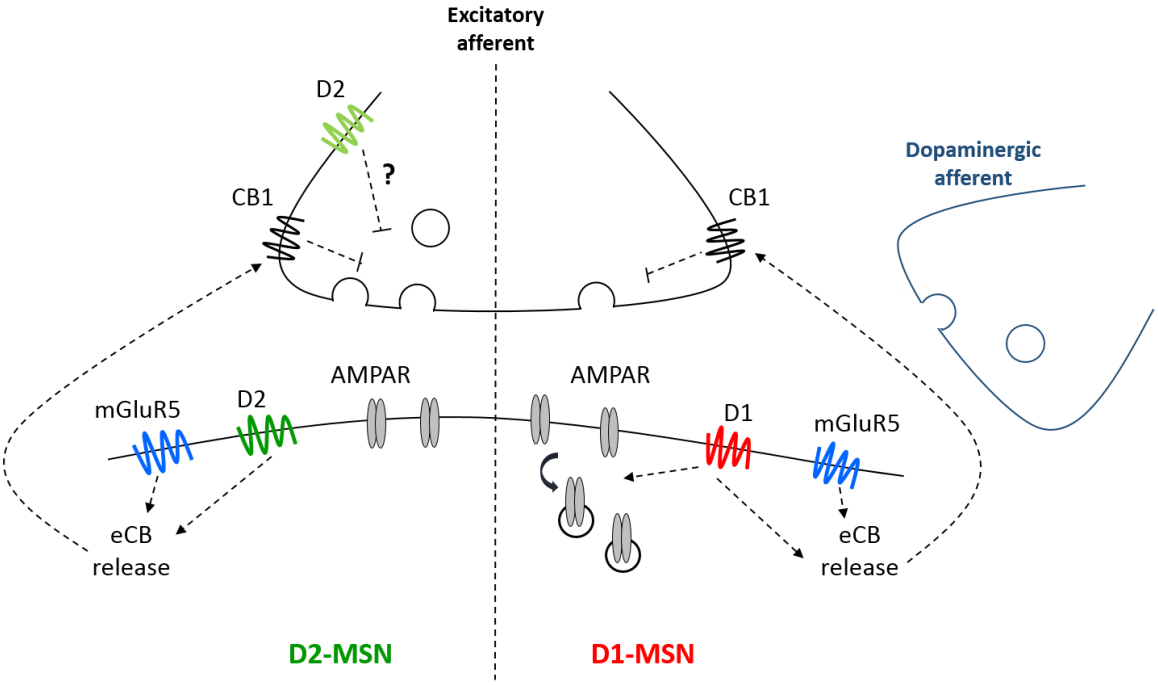

Figure 5. Summary schematic of cellular signaling mechanisms altered by cocaine experience. Distinct mechanisms promote plasticity at D1- vs. D2-MSN synapses in the NAc. We found that activity of mGluR5 receptors is necessary for inducing depotentiation of augmented post-synaptic AMPAR-mediated transmission during abstinence from cocaine, potentially by promoting internalization of AMPARs. In specific MSN cell types, we identified that D1 receptors are capable of altering AMPAR-signaling at synapses on D1-MSNs, likely also reflecting alterations in AMPAR trafficking mechanisms in these cell types. Additionally, CB1 signaling at both D1- and D2-MSN synapses was necessary for the suppression of mEPSC frequency induced by cocaine re-exposure, suggesting that eCB-mediated reductions in synaptic strength do not necessarily occur selectively at D2-MSN synapses. Furthermore, we found that activation of CB1 receptors downstream of dopamine receptor activity mediates the dopamine-induced reduction in mEPSC frequency at both MSN cell types. Thus, cellular signaling factors (i.e. mGluR5 and DA receptors) activated by cocaine re-exposure may converge on common factors including eCB signaling mechanisms to promote a reduction in synaptic strength.

Throughout these studies, we consistently observed complex alterations in presumed measures of pre-synaptic plasticity (i.e. mEPSC frequency), indicating that cocaine re-exposure may have complex effects on terminal release properties by potentially engaging receptors expressed on glutamatergic afferents that regulate release, including CB1 and D2 receptors.

Alterations in glutamatergic synaptic transmission induced by experience with cocaine may therefore profoundly alter the responsiveness of MSNs to future stimulation by dopamine, modifying the capacity of dopamine to effectively gate activity and plasticity at glutamatergic synapses. Additionally, changes in dopamine receptor expression and signaling mechanisms (Anderson and Pierce, 2005) may modify how dopamine and glutamate receptor signaling mechanisms converge and interact at an intracellular level. Furthermore, mGluR1/5 and eCBs may additionally adjust MSN responsiveness to alterations in glutamatergic signaling induced by cocaine experience. While future work is needed to characterize how particular inputs impinging on the NAc as well as specific neural ensembles within the NAc differentially mediate plasticity, this work begins to characterize how cocaine experience alters common signaling factors that ultimately drive addiction-related behavior.

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